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The effect of i.m. administered human butyrylcholinesterase (HuBuChE) on the i.v. bolus and inhalation toxicokinetics of (±)-sarin, C(±)P(±)-soman and percutaneous toxicokinetics of VX in guinea pigs was studied. After i.m. administration of HuBuChE, the BuChE activity in blood gradually increased, reaching a maximum after 20-24 h and remaining stable for about another 24 h. Approximately 25% of the administered dose of HuBuChE was circulating in blood at 24 h after the i.m. injection. It was decided to study the effect of HuBuChE on the toxicokinetics of nerve agent at 24 h after administration of the enzyme. Pretreatment with HuBuChE (at a molar equivalent of 1.4 LD50 (i.v.) of C(±)P(±)-soman in the guinea pig) resulted in a significant decrease of the AUC of the various toxicokinetic curves. Effect of pretreatment of HuBuChE was also studied in guinea pigs that were nose-only exposed to soman and sarin and percutaneously to VX. HuBuChE pretreatment resulted in a significant decrease of the AUC of the toxicokinetic curves of soman but not of sarin. VX was not detected in blood of hairless guinea pigs that were percutaneously exposed to VX. A physiologically based pharmacokinetic model was developed for the toxicokinetics of soman in naive and HuBuChE-pretreated animals.
Furthermore, via fluoride-induced reactivation a significantly higher concentration of (±)-sarin bound in blood was demonstrated in these animals.

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I INTRODUCTION

An effective therapy against intoxication with nerve agents has been developed involving combined administration of atropine, oxime and anticonvulsant. Administration of the therapeutic agents almost immediately after intoxication is a prerequisite for their efficacy, which renders this approach less satisfactory under field conditions. The treatment is *a fortiori* much less effective if the nerve agent forms an inhibited acetylcholinesterase (AChE) that is, or rapidly becomes, resistant to reactivation by an oxime. Furthermore, even if the treatment would protect against the lethal effects of nerve agent intoxication, the combined administration of atropine, oxime and anticonvulsant does not prevent the occurrence of post-exposure incapacitation.

Additional pretreatment with carbamates, such as pyridostigmine and physostigmine, has only partly solved these problems. The protection offered by carbamates is based on reversible inhibition of AChE, which at the same time shows the limits of pretreatment with inherently toxic compounds. The requirement for the application of a sign-free dose is contradictory to aiming at complete protection. Furthermore, the peripherally acting pyridostigmine, which is in use as the standard pretreatment drug, does not protect against post-exposure incapacitation. Application of the centrally acting physostigmine may prevent incapacitating effects of nerve agent intoxication but on the other hand will increase the possibility of adverse effects on the military performance of individuals not exposed to any nerve agent.

Therefore, attention has been paid to pretreatment with highly reactive scavengers, which would intercept or destroy the nerve agent before it could reach its target site, when entering the blood stream. It may be expected that effective scavengers offer protection against both lethal and incapacitating effects of an acutely toxic dose. In addition, if a scavenger remains in circulation at an effective concentration during a relatively long period of time, the pretreatment will, *a fortiori*, protect against a long term exposure to low doses of a nerve agent (Benschop *et al.*, 1998). It is further anticipated that bioscavengers do not induce adverse physiological effects, particularly when bioscavengers from human origin are applied.

As early as 1957, Cohen and Warringa achieved some protection in rats against a lethal subcutaneous dose of diisopropyl phosphorofluoridate and sarin by pretreatment with an enzyme capable of hydrolyzing organophosphates. In more recent years, the feasibility of using bioscavengers that can rapidly bind nerve agents has been studied, such as monoclonal antibodies (Lenz *et al.*, 1984; Brimfield *et al.*, 1985), fetal bovine serum AChE (Wolfe *et al.*, 1987; Ashani *et al.*, 1991; Maxwell *et al.*, 1992; Wolfe *et al.*, 1992) and human plasma butyrylcholinesterase (HuBuChE) (Ashani *et al.*, 1993; Raveh *et al.*, 1997; Allon *et al.*, 1998).

Very promising results were obtained with HuBuChE as a scavenger. The enzyme is rapidly distributed in laboratory animals, such as mice, rats, guinea pigs, and rhesus monkeys, after i.v. administration, followed by a slow elimination (Ashani *et al.*, 1993). In addition, the enzyme is sufficiently absorbed following an i.m. administration to provide therapeutically significant blood levels over 10 – 70 h in laboratory animals, which is a prerequisite for practical application. The peak level in blood after i.m. administration amounted to 50-60 % of the concentration obtained immediately after i.v. administration of the same amount of enzyme (Ashani *et al.*, 1993). Pretreatment with the enzyme resulted not only in an increase in survival of mice, rats and rhesus monkeys intoxicated (i.v.) with C(±)P(±)-soman or other nerve agents, but also in a significant alleviation of post-exposure incapacitation. An effective protection of

guinea pigs against respiratory exposure to C(±)P(±)-soman has recently been reported (Allon *et al.*, 1998). Moreover, since the efficacy of HuBuChE as a scavenger is based on the inhibitory properties of the challenging agent, it can be expected that such scavengers will be effective against nerve agents having a wide variety of chemical structures.

The final aim is the use of this enzyme as a scavenger for protection of humans against acute intoxication by nerve agents. Although the results obtained in laboratory animals indicate the possible usefulness of HuBuChE as a scavenger, the information reported so far is insufficient for a thorough and quantitative description of the protective mechanism. Consequently, a reliable extrapolation of the working mechanism to human beings is not yet possible, although this will be needed for further development of the enzyme as a pretreatment drug for application in humans. Additional information is needed on at least three aspects that are elucidated in the following.

A. Toxicokinetics of nerve agents
The effect of nerve agent intoxication on the scavenger activity in blood of laboratory animals has been reported (Ashani *et al.*, 1993; Allon *et al.*, 1998). In addition to the decrease of HuBuChE activity in blood, the time course of the other reactant, i.e., the nerve agent, is essential in order to describe the interaction between the enzyme and the nerve agent adequately. These experiments will answer the key question with regard to the acceleration of nerve agent scavenging by HuBuChE down to levels that are toxicologically no longer relevant.

B. Stereoselectivity of HuBuChE inhibition
The common nerve agents are chiral organophosphates. The stereoisomers of these compounds may differ largely in their anti-butyrylcholinesterase activities. Therefore, rate constants for inhibition of HuBuChE by the single stereoisomers should be available. In contrast with AChE (Benschop and De Jong, 1988), these parameters are hardly known for inhibition of BuChE.

C. Binding in extravascular compartments
After i.m. administration, the enzyme will be distributed between the peripheral compartments and the central compartment prior to intoxication in the protection experiments. Furthermore, a fraction of the nerve agent may escape the scavenger circulating in the blood. Therefore, information on the fate of HuBuChE in extravascular compartments and of the relative importance of (additional) binding sites is needed for a thorough description of the protective mechanism of the scavenger.

The proposed studies should provide data that is lacking for the three above-mentioned items. Our proposed studies will be focused on C(±)P(±)-soman, but some investigations on (±)-sarin and (±)-VX will also be performed.

Ad A. Toxicokinetic studies
During the last decade, we have performed various studies on the toxicokinetics of the stereoisomers of nerve agents in rat, guinea pig and marmoset (Benschop and De Jong, 1991; Benschop *et al.*, 1995, 1998; Due *et al.*, 1993, 1994; Langenberg *et al.*, 1998; Spruit *et al.*, 2000; Van der Schans *et al.*, 2000). For this purpose, we developed analytical procedures which allow the quantitation of nerve agent stereoisomers in blood and tissues at levels down to and below those of toxicological relevance. An estimation of a toxicologically relevant level was based on the rationale (Benschop *et al.*, 1987) that only a small fraction (5-10 %) of active AChE has to be reactivated, e.g., by oximes, after total inhibition by a nerve agent in order to sustain vital

functions. A nerve agent concentration that can re-inhibit this fraction, will have a toxicologically relevant effect.

One of the conclusions from these studies is that from a toxicokinetic point of view the guinea pig is a better model for a primate than the rat. The studies performed in guinea pigs include determinations of time courses of the stereoisomers in blood after administration of supra-lethal i.v. bolus doses of C(±)P(±)-soman and after administration of sub-lethal doses of C(±)P(±)-soman and (±)-sarin both by an i.v. bolus injection and by respiratory exposure. Studies in which guinea pigs were exposed nose-only for a long period of time (300 min) to a low level of C(±)P(±)-soman (20 ppb) were also included. Short term and long term nose-only exposure to C(±)P(±)-soman and (±)-sarin in guinea pigs are routinely performed in our laboratory (Benschop *et al.*, 1995; Benschop *et al.*, 1998; Langenberg *et al.*, 1998; Spruit *et al.*, 2000), whereas the same technique was applied to expose marmosets after small modification of the exposure chamber (Van Helden *et al.*, 2000, 2003). Studies on the toxicokinetics of the stereoisomers of (±)-VX after a lethal i.v. bolus in hairless guinea pigs and marmosets and a lethal percutaneous dose in hairless guinea pigs were recently concluded (Van der Schans *et al.*, 2000) within the context of Cooperative Agreement DAMD17-97-2-7001.

The results obtained from the abovementioned toxicokinetic studies were intended to serve as reference values for the newly proposed investigations on the efficacy of HuBuChE as a nerve agent scavenger.

We proposed to protect the animals by i.m. administration of HuBuChE, since this route is much more appropriate than i.v. administration for application of a scavenger in a realistic scenario. The ratio of the dose of HuBuChE relative to the dose of the nerve agent was chosen on the basis of results reported by Ashani *et al.* (1993) and Allon *et al.* (1998) in order to obtain sufficient protection in a similar experiment. The challenge dose of the nerve agent was chosen to be twice a lethal dose, in accordance with the experimental setup of most of the experiments reported by Ashani and coworkers. Moreover, in recent experiments on therapeutic efficacy of oximes (Pearce *et al.*, 1999), protection against twice the lethal dose has been accepted as a realistic goal.

Three series of toxicokinetic studies are performed. In the first series of experiments, the toxicokinetics of the stereoisomers of C(±)P(±)-soman, (±)-sarin and (±)-VX are studied following an i.v. bolus administration of the agent in guinea pigs pretreated with HuBuChE. In these experiments the total amount of nerve agent entering the animals will be known accurately, which is important for a reliable description of the protective mechanism of the scavenger. In addition, toxicokinetics will be studied after a repeated i.v. dose of C(±)P(±)-soman (2 LD50) in order to expand the dose range for which to obtain data on the protective activity of the scavenger.

In the second series of toxicokinetic studies guinea pigs are exposed to the nerve agents by the most probable way of intoxication when used under realistic conditions, i.e., exposure via inhalation for the more volatile agents C(±)P(±)-soman and (±)-sarin and via the skin for the nerve agent (±)-VX. The toxicokinetics of the stereoisomers of C(±)P(±)-soman and (±)-sarin will be studied following short term (2 min) nose-only exposure of pretreated guinea pigs to these two agents at doses corresponding with 2 LCt50. In order to investigate the efficacy of HuBuChE scavenger in long term-low level exposure to nerve agents, the time course of the stereoisomers of C(±)P(±)-soman will also be determined during and after a 300-min exposure

of HuBuChE-pretreated guinea pigs to 2 LCt50 of C(±)P(±)-soman. Furthermore, the toxicokinetics of the stereoisomers of (±)-VX will be studied after percutaneous administration of 2 LD50 of the agent to HuBuChE-pretreated hairless guinea pigs.

Extrapolation to humans is more reliable if toxicokinetic data obtained in non-human primates are available. Therefore, a third, but limited, study will be performed on the toxicokinetics of the stereoisomers of C(±)P(±)-soman following respiratory exposure of HuBuChE-pretreated marmosets to the agent. As a reference, the toxicokinetics will be studied in non-protected animals challenged with 0.8 LCt50 of C(±)P(±)-soman. The LCt50 value for C(±)P(±)-soman in marmosets will be calculated as the estimated LCt50 values for C(±)P(±)-soman in humans (Black and Harrison, 1996) corrected for marmosets by multiplying with the ratio between the value for (±)-sarin in monkeys (Marrs *et al.*, 1996) and in humans (Black and Harrison, 1996).

Ad B. Stereoselectivity of HuBuChE inhibition
Rate constants for inhibition of HuBuChE by the stereoisomers of C(±)P(±)-soman are determined by using the single stereoisomers at first-order or second-order conditions, according to common procedures. The stereoisomers are isolated according to procedures well-known in our laboratory (Benschop *et al.*, 1984). The inhibition reactions with (±)-sarin and (±)-VX will be performed by using the racemic mixtures of the nerve agents. Kinetic analysis of the inhibition of HuBuChE performed at second-order conditions will allow to derive the rate constants for the individual stereoisomers (Boter and Van Dijk, 1969).

Ad C. Binding in extravascular compartments
Indications for binding of the nerve agent to extravascular HuBuChE in pretreated guinea pigs intoxicated by C(±)P(±)-soman were obtained from preliminary studies using a physiologically based model which describes the toxicokinetics of C(±)P(±)-soman after i.v. administration in guinea pigs. This model has been developed as a cooperative effort of TNO Prins Maurits Laboratory and U.S. Army Medical Research Institute of Chemical Defense (Langenberg *et al.*, 1997). Recently, we performed preliminary calculations on the influence of HuBuChE scavenger on the toxicokinetics of C(±)P(±)-soman and on the inhibition of the scavenger (De Jong *et al.*, 2000). The rate constants for reaction of the C(±)P(±)-soman stereoisomers with the scavenger were estimated from the overall rate constant for inhibition of HuBuChE by C(±)P(±)-soman (Ashani *et al.*, 1993) and from the relative potencies of the stereoisomers as determined for horse serum BuChE (Keijer and Wolring, 1969). The dose of i.v. administered C(±)P(±)-soman corresponded with 2 LD50, whereas the dose of scavenger was taken from Ashani's study (1993) yielding complete protection. Assuming that the scavenger circulated *only* in the central compartment, the model predicts that the residual enzyme activity is very low (< 5%), whereas the observed HuBuChE activity in rats and rhesus monkeys is relatively high (40-45 %).

We decided to perform the following three series of experiments in order to obtain information on the effect of nerve agent administration on the fate of HuBuChE in blood and in extravascular compartments of the guinea pig.
In the first series, the distribution of HuBuChE over blood, lung, liver, kidney and brain is determined at the point of time after i.m. administration to guinea pigs at which the nerve agent is administered in the toxicokinetic experiments. HuBuChE concentrations in blood and in homogenates of the selected tissues will be determined on the basis of enzyme activity.
In the second series, samples of the same tissues of guinea pigs pretreated with the enzyme (i.m.) are taken 1 min after i.v. administration of a dose corresponding with 2 LD50 of C(±)P(±)-^{14}C-soman (labeled in the phosphonomethyl group). It will be attempted to separate inhibited (and

non-inhibited) HuBuChE from other binding sites to which C(±)P(±)-^{14}C-soman has been bound in tissues and in blood by means of affinity chromatography on procainamide sepharose (Ashani *et al.*, 1993). Preliminary experiments in our laboratory have shown that elution of C(±)P(±)-soman-inhibited HuBuChE from the gel is retarded in a similar way as elution of the non-inhibited enzyme. The collected fraction will be tested for radioactivity and HuBuChE activity. The third series comprises similar experiments, but the determinations will be carried out in samples taken 90 min after C(±)P(±)-^{14}C-soman administration.

Modeling studies

Finally, information obtained on the rate constants for inhibition of HuBuChE by the stereoisomers of C(±)P(±)-soman and on the distribution of the enzyme over various organs will be introduced into the physiologically based model for the i.v. toxicokinetics of C(±)P(±)-soman in the guinea pig (Langenberg *et al.*, 1997; De Jong *et al.*, 1993, 1995, 2000). The model will be validated by comparing simulated levels of the toxic C(±)P(-)-soman isomers in blood and of C(±)P(±)-soman bound to HuBuChE after an i.v. bolus administration of the nerve agent with the levels determined in the proposed experiments.

II. EXPERIMENTAL PROCEDURES

II.1 Materials

(±)-Sarin (isopropyl methylphosphonofluoridate), C(±)P(±)-soman (1,2,2-trimethylpropyl methylphosphonofluoridate), C(+)P(±)-soman, C(-)P(±)-soman, (±)-VX [(O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothioate)], and d7-(±)-sarin, d13-C(-)-P(+)-soman and V33 [(O-n-propyl S-(2- diisopropylaminoethyl) methylphosphonothioate)] were obtained from the stocks at TNO Prins Maurits Laboratory.

Human Butyrylcholinesterase was obtained from Dr. Doctor from Walter Reed Army Institute of Research (Washington DC). The enzyme, dissolved in glycerol, was transferred into phosphate buffered saline (PBS) via dialysis (4 °C, overnight), after which the volume was reduced by leading the solution through a 30 kD cut-off filter (Schleicher & Schuell, Keene, NH, USA). If the enzyme was received in the lyophilized form, the enzyme was dissolved in an appropriate volume of saline.

Dormicum® was purchased from Roche (Mijdrecht, The Netherlands), Hypnorm® from Solvay-Duphar (Amsterdam, The Netherlands), Vetranquil® and Nembutal® from Sanofi (Maassluis, The Netherlands), and heparin, veronal sodium and atropine sulfate from Brocacef (Rijswijk, The Netherlands).

Analytical grade ethyl acetate, 2-propanol, n-hexane, methanol, acetone, acetic acid, sodium acetate, sodium chloride, sodium hydroxide, disodium hydrogen phosphate, potassium dihydrogen phosphate, aluminum sulfate, potassium fluoride, sodium dodecylsulfate, α-chymotrypsin and saponin were obtained from Merck (Darmstadt, Germany). Butyrylthiocholine iodide and 5,5'-dithiobis(2-nitrobenzoic acid) were purchased from Aldrich (Brussels, Belgium). Rabbit serum was procured from Harlan NL (Horst, The Netherlands).

Nexus® solid phase extraction cartridges were purchased from Varian (Middelburg, The Netherlands). Extrelut 20 was procured from Merck.

II.2 Methods

II.2.1 Activity of butyrylcholinesterase in blood

Blood samples were diluted tenfold with a solution of 1% saponin in 100 mM phosphate buffer (pH 7.5). Subsequently, the diluted samples were frozen in liquid nitrogen if they were not analyzed immediately. BuChE activity was measured according to the colorimetric method described by Ellman *et al.* (1961), using 0.4 mM butyrylthiocholine iodide as a substrate and 0.4 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 0.05 M phosphate buffer, pH 8.0 at 25 °C. The activity of BuChE was calculated using the following formula:

Activity = $A_{412\,nm}$* V / ε * t* v, in which $A_{412\,nm}$= absorbance at 412 nm, V is reaction volume (mL), ε = molar extinction coefficient, 13.96 mM^{-1}, t = reaction time (min), v is sample volume mL. The concentration of the enzyme can be calculated according to the rule that 60 U of enzyme is equal to 1 nmol. (Grunwald *et al*, 1997)

II.2.2. Sample preparation of (±)- sarin from blood

Blood samples (1 part) were mixed with three parts of stabilization buffer (0.2 M sodium acetate, pH 3.5, 1.6 mM aluminum sulfate, 1 µg C(±)P(±)-soman/mL), after which the internal standard (d7-(-)-sarin in 2-propanol) was added. The mixture was transferred onto a Nexus® solid phase extraction cartridge (200 mg) and eluted with 2 mL ethyl acetate. The ethyl acetate layer was separated from the aqueous layer by freezing the aqueous layer with a dry ice/acetone mixture. The ethyl acetate phases were transferred onto Tenax for analysis with the GLC-configuration described in paragraph II.2.6.

II.2.3 Sample preparation of C(±)P(±)-soman from blood

Blood samples (1 part) were mixed with three parts of stabilization buffer (0.2 M sodium acetate, pH 3.5, 1.6 mM aluminum sulfate, 1 µg of neopentyl sarin/mL), after which the internal standard d13-C(-)P(+)-soman was added. The mixture was transferred onto a Nexus® solid phase cartridge (200 mg) and eluted with 2 mL ethyl acetate. The ethyl acetate layer was separated from the aqueous layer by freezing the aqueous layer with dry-ice/acetone mixture. The ethyl acetate phases were transferred onto Tenax tubes for analysis with the GLC configuration described in paragraph II.2.7.

II.2.4 Sample preparation of (±)-VX from blood

Blood samples were mixed with internal standard, V33 [O-n-propyl S-(2-diisopropylaminoethyl) methylphosphonothioate]. Next the mixture was made alkaline with sodium hydroxide. A volume of 30 µl 1 M NaOH was needed to increase the pH of a 250 µl blood sample to levels between 10.5 and 11. Next the mixture was twice extracted with two volumes of a mixture n-hexane:methanol (95:5, v:v). The two organic layers were pooled and the solvent was evaporated with a gentle stream of nitrogen. The residue was dissolved in 80 µl of n-hexane and analyzed with GC-NPD as described in II.2.8.

II.2.5 Fluoride-induced reactivation of organophosphates bound to esterases in blood

The phosphyl moiety of sarin, soman and VX as bound to esterases and probably other binding sites in blood was reactivated with fluoride ions according to the procedure as described by Polhuijs *et al.* (1997). The procedure was slightly modified, i.e., the Sep-Pak C18 cartridge was replaced a 200-mg Nexus® SPE-cartridge, which provided for a higher and more reproducible recovery of (±)-sarin. The ethyl acetate eluates from the Nexus® cartridges were transferred onto Tenax for analysis with the GLC-configuration described in paragraphs II.2.6, II.2.7 and II.2.9.

II.2.6. Gas chromatographic analysis of (±)-sarin

The gas chromatographic system used to analyze the stereoisomers of (±)-sarin consisted of a Carlo Erba HRGC 5160 Mega Series (Milan, Italy), equipped with an alkali flame detector (NPD, Carlo Erba), a thermodesorption cold trap injector (TCT, Chrompack, Middelburg, The Netherlands), and a Chrompack MUSIC system (Multiple Switchable Intelligent Controller) for two-dimensional gas chromatography.

The desorption tubes (length 15 cm, i.d. 2.5 mm) were partly (ca. 80 %) filled with Tenax TA 60-80 mesh (Varian). A glass wool plug was firmly pushed on top of the Tenax material and was fixed with a metal clamp. The tubes were preconditioned by heating under a stream of nitrogen at 300 °C for at least 4 h.

The pre-column of this system was CPSil 8 CB, (length 30 m i.d. 0.53 mm, film thickness 5 µm). The analytical column was a CP-Cyclodextrin-B2,3,6-M-19 column (Chrompack, length 50 m, 0.25 mm i.d., film thickness 0.25 µm).

Tenax tubes were loaded with sarin samples in ethyl acetate (400 µl) in portions of 100 µl. In between each loading the tube was flushed with nitrogen (240 mL/min) for three min. Next, the tube was placed in the thermodesorption device and sarin was desorbed by heating the tube for six min at 200 °C. Sarin was focused in a cold trap which was cooled down to –60 °C with liquid nitrogen. The cold trap consisted of 1 m CPSil 8 CB (i.d. 0.53 mm). Flow (helium) through the pre-column was 16 mL/min. Cooling was started before the tube was placed. Sarin was injected onto the pre-column by flash heating from –60 °C to 220 °C at a rate of 21 °C/sec; the temperature of the TCT-injector was maintained for 10 min. The effluent of the pre-column was monitored with an FID detector. The effluent of the pre-column within the time interval of 7.1 and 9.7 min was focused in a second cold trap, which was cooled with liquid carbon dioxide,

controlled by the MUSIC system. Subsequently, sarin was injected onto the analytical column. The flow through the analytical column was pressure-controlled and was kept at 106 kPa. Overall oven temperature program of the GC: Initial temperature 70 °C maintained for 6 min, raised to 90 °C at a rate of 10 °C/min, cooled down to 70 °C at infinite rate, maintained at 70 °C for 5 min and raised to 82 °C at a rate of 0.5 °C/min. Next, the temperature was raised to 200 °C and maintained for 5 min. The detector base temperature was kept at 250 °C. Make-up gas for the NP-detector was helium at a flow-rate of 38 mL/min. Flow-rates of air and hydrogen through the NP-detector were 350 and 35 mL/min, respectively.

II.2.7 Gas chromatographic analysis of C(±)P(±)-soman

Chiral gas chromatographic analyses of soman were performed on a gas chromatography instrument (Agilent) equipped with flameless NPD detector, FID detector, a multicolumn switching device (Gerstel, Muhlheim a/d Ruhr, Germany), a cooled injection system (CIS), a cold trap (CTS) and an automated thermodesorption device (TDSA, Gerstel). The pre-column was a CPSil 19 (33 m x 0.32 mm x 1 µm), the second analytical column was a ChiraSil Val-L column (50 m x 0.25 mm x 0.4 µm). Soman samples in ethyl acetate (max. 400 µl) were loaded in portions of 50 µl on Tenax filled glass tubes and subsequently purged with a flow of nitrogen (50 mL/min for 20 min). After installing the tube in the thermodesorbing device, the system was started by heating (60 °C/min) the desorber from 30 °C to 200 °C. The transport from the desorber to the CIS was under splitless conditions, which means that all components desorbed from the Tenax material are transported to the CIS. During this process the CIS is cooled with liquid nitrogen (-50 °C) and runs in the solvent vent mode. (Flow is 50 mL/min, pressure 15 kPa). After the desorption program the CIS is kept cool for 0.02 min followed by a splitless injection by flash heating with a rate of 12 °C/s to 250 °C and maintaining this temperature for 2 min. The pressure on the first column is 240 kPa. The pressure at the cross joint between precolumn and analytical column is maintained at 100 kPa. The effluent of the first column drains off through a mass flow controller which is set at 10 mL/min. The oven program of the first chromatography step is 100 °C→5° C/min → 140 °C. The soman isomers leave the first column between 7.8 and 9 min. During this time the flow through the mass flow controller is switched off and the counter pressure of 100 kPa at the switching point is turned off as well. The soman isomers migrate into the cold trap which is mounted after the cross joint just before the analytical column. During the trapping period from 7.8 until 10.7 min the cold trap is cooled with liquid nitrogen to -80°C. During the trapping process the oven temperature is cooled down to 90 °C. Next the cold trap is heated at a rate of 25 °C/s to 240 °C. In the same time the pressure at the inlet of the analytical column is set at 160 kPa and the flow through the mass flow controller is restored to 10 mL/min. The oven program during the second chromatography stage is 90 °C → 1.5 °C/min → 130 °C(1') →30 °C/min →180 °C (1'). The FID operates at 250 °C. Gas flows are 30 mL hydrogen /min, 350 mL air /min and 35 mL helium /min. The flameless NPD operates at 300 °C. Gas flows are 3 mL hydrogen/min, 60 mL air /min and 4 mL helium /min. The bead voltage for the NPD is set at 3.5 V.

II.2.8 Gas chromatographic analysis of (±)-VX

Chromatographic conditions for the quantitative analysis of (±)-VX were roughly the same as described by Bonierbale *et al.* (1996). The gas chromatography system consisted of a HRGC Mega II 8560 instrument (Fisons Instruments, Milano, Italy) equipped with a nitrogen-phosphorus detector (detector nr.15 Carlo Erba Milano, Italy), a split-splitless and an on-column injection port, and an AS 800 autosampler. The detector temperature was maintained at 300 °C. Injections were performed using the splitless injection mode. The injector temperature was maintained at 260 °C. The split valve was closed for 60 s during the injection. The injection

volume ranged between 2-8 µl. Chromatographic conditions comprised a CPSil 8 column with a length of 25 m, an internal diameter of 0.25 mm and a film thickness of 1.2 µm (Chrompack-Varian, Middelburg, The Netherlands). The oven temperature was 100 °C at the start, which was maintained for 2 min, after which the temperature was raised to 200 °C at a rate of 25 °C/min, which was maintained for 5 min. Next, the oven temperature was elevated to 300 °C at a rate of 25 °C /min, which was maintained for 15 min. After the analysis the oven was cooled down at infinite rate to 100 °C. The carrier gas flow (helium) was 2.5 mL/min. Detector gas flows were 35, 350 and 37 mL/min for hydrogen, air and helium, respectively. Data were acquired and analyzed with ChromCard for USB software v2.2 (Carlo Erba, Milano, Italy).

II.2.9 Gas chromatographic analysis of ethyl sarin

Analyses were performed on an Agilent 6890A gas chromatography instrument coupled with an Agilent 5973 Mass Selective Detector operating in the positive ion chemical ionization mode with ammonia as reaction gas. The capillary column was CP VF-5MS (30 m x 0.25 mm x 1.0 µm) Initial flow of helium gas was 1.0 mL/min. Oven program: initial 60 °C maintained for 2 min, rising at 25 °C/min and maintained at 280 °C for 4.2 min. Injections were performed in the splitless mode (60s). The temperature of the injector was 250 °C, whereas the temperature of the transfer line was 270 °C. The following masses were acquired: 144 for ethyl sarin $[M\text{-}NH_4]^+$ and 149 for d5-ethyl sarin $[d_5\text{-}M\text{-}NH_4]^+$.

II.2.10 Isolation of individual nerve agent stereoisomers

II.2.10.1 Isolation of (-)-d7-sarin from (±)-d7-sarin

A solution of (±)-d7-sarin in 2-propanol (100 µl) was incubated in 12 mL of rabbit serum for 30 s at 37 °C. Next the mixture was poured onto a bed of Extrelut 20 (7.5 g). After absorbing the fluid, the bed was rinsed with 35 mL of ethyl acetate. Ethyl acetate containing (-)-d7-sarin was recovered. Its concentration was determined with the GLC-configuration described in II.2.6.

II.2.10.2. Isolation of soman isomers

Soman isomers were isolated analogously to methods described by Benschop *et al.* (1984). GC analysis on Chirasil-Val (see II.2.7) was used to quantify the concentrations of the isomers in the final solution and to check their optical purities.

Isolation of C(-)P(-)-soman from C(-)P(±)-soman

C(-)P(±)-soman (10 mg) was incubated in rabbit serum (25 mL) for 50 s at 37 °C. Next, the serum was poured onto a bed of Extrelut 20 (25 g). After the fluid being absorbed by the Extrelut, the soman isomer was extracted with 70 mL of ethyl acetate. The recovered ethyl acetate was evaporated under reduced pressure (44-50 mm Hg) to a residual volume of approx. 50 µl and diluted to a final volume of 1 mL with acetonitrile. Chiral GLC-analysis revealed that this preparation of C(-)P(-)-soman contained *ca.* 2% of the C(+)P(-)-soman isomer.

Isolation of C(-)P(+)-soman from C(-)P(±)-soman

C(-)P(±)-soman (10 mg) was incubated in 35 mL phosphate buffer (0.01 M, pH 6.2) containing 1.75 g of α-chymotrypsin. After 60 s of incubation the mixture was poured onto a bed of 30 g of Extrelut 20. After equilibration, the bed was rinsed with 110 mL of ethyl acetate. The recovered ethyl acetate was evaporated in a rotary evaporator under reduced pressure (45-50 mm Hg) to a residual volume of approx. 100 µl. Next, the solution was diluted to 1 mL with acetonitrile. Chiral GLC-analysis revealed an optical purity of this C(-)P(+)-soman preparation of 99.9%.

Isolation of C(+)P(-)-soman from C(+)P(±)-soman
C(+)P(±)-soman (10 mg) was incubated for 25 s in 12.5 mL of rabbit serum at 37 °C. The mixture was poured onto a bed of 7.5 g of Extrelut 20. After equilibration the bed was rinsed with 37.5 mL of ethyl acetate. The recovered ethyl acetate was evaporated in a rotary evaporator under reduced pressure (45-50 mm Hg) to a residual volume of *ca.* 100 µl. Next, the solution was diluted to a final volume of 1 mL with acetonitrile. Chiral GLC-analysis revealed an optical purity of this C(+)P(-)-soman preparation of 99.7%.

Isolation of C(+)P(+)-soman from C(+)P(±)-soman
C(+)P(±)-soman (10 mg) was incubated in 40 mL phosphate buffer (0.01 M, pH 6.2) containing 3.48 g of α-chymotrypsin. After 60 s the mixture was poured onto a bed of 40 g of Extrelut 20. After absorption of the fluid, the bed was rinsed with 110 mL of ethyl acetate. The recovered ethyl acetate was evaporated in a rotary evaporator under reduced pressure (45-50 mm Hg) to a residual volume of *ca.* 100 µl. Next, the solution was diluted to 1 mL with acetonitrile. Chiral GLC-analysis revealed an optical purity of this C(+)P(+)-soman preparation of 99.9%.

II.2.11 Determination of rate constant of inhibition of HuBuChE by the stereoisomers of the nerve agents(±)-VX, (±)-sarin and C(±)P(±)-soman
HuBuChE was dissolved in 50 mM veronal buffer, pH 7.4, containing 0.1 M sodium chloride, to the desired concentration. Inhibition was started by addition of a small volume (sarin, VX) or an equal volume (soman isomers) of a solution of the organophosphate at the desired concentration in the same buffer. Aliquots (30-60 µl) were taken at various times of incubation at 38 °C and added to a solution (4 mL) of 0.4 mM butyrylthiocholine iodide and 0.4 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 0.05 M phosphate buffer, pH 8.0. After reacting at 25 °C for 10 min [(±)-sarin, (±)-VX] or 20 min (C(±)P(±)-soman stereoisomers), the substrate hydrolysis was stopped by addition of a solution of 13% sodium dodecylsulfate in water (0.15-0.20 mL). Subsequently, the extinction of the solution was measured at 412 nm after approximately 3 min.

II.2.12 Animals
Male albino outbred guinea pigs of the Dunkin-Hartley type (species identification: Crl:(HA)BR), weighing 350-400 g were purchased from Harlan NL (Horst, The Netherlands). Hairless guinea pigs (species identification: Crl:IAF(HA)BR) were purchased from Charles River (Maastricht, The Netherlands). Health certificates were examined before delivery was approved and were subsequently archived. The animals were housed in temperature- and humidity-controlled rooms. They were allowed to eat and drink *ad libitum*. Teklad® guinea pig food was procured from Harlan NL. Analysis reports of the food batches were received, inspected and filed. Two standard operation procedures were applicable to the care of the guinea pigs, i.e., 'Ordering and Housing of Experimental Animals' (SOP Q213-W-039) and 'Cleaning and Maintenance of Animal Facilities' (SOP Q213-W-040). The animals were allowed to acclimatize to their new environment for at least 1 week before they were used in any experiment. Mostly, the guinea pigs were in the weight range of 500-700 g when they were used in the experiments.
Male and female marmosets (*Callithrix jacchus*), 1½-2 years of age, were purchased from Harlan NL (Horst, The Netherlands). A health certificate was obtained for each animal prior to delivery. In accordance with SOP Q215-W-058 ('Biotechnique of marmoset monkeys') the animals were individually housed in stainless steel wire cages in a temperature (25 °C) and humidity (50 %) controlled room. They were allowed to drink *ad libitum*. Feeding was according to the aforementioned SOP. The animals were allowed to acclimatize to their new environment for at least 1 week before they were used in any experiment. The protocol for the animal

experiments was approved in August 2000 by the TNO Animal Experiment Committee under number DEC 834 and extended in February 2004.

II.2.13 Administration of HuBuChE

At 24 h before the start of a toxicokinetic experiment, guinea pigs were injected i.m. in the hind legs with a dose of HuBuChE dissolved in phosphate buffered saline (PBS). The maximum volume injected was 600 µl, divided over both hind legs. The dose of HuBuChE depended on the type of experiment (i.v. bolus or nose-only exposure) and the weight of the animal.
In pharmacokinetic experiments, guinea pigs were anesthetized and a carotid cannula was inserted which protruded from the neck. Heparin (60 U) was administered via the cannula. Blood samples were drawn via the cannula just prior to administration of HuBuChE and at various time points after i.m. administration of this scavenger. At the end of the experiment the animals were euthanized with an overdose of Nembutal® (0.4 mL, i.p.).
In other pharmacokinetic experiments marmosets were i.m. injected in the hind leg with BuChE dissolved in PBS. Blood samples were drawn by means of a heel prick. At the end of the experiment the animals were euthanized with an overdose of Nembutal® (0.4 mL, i.p.). In toxicokinetic experiments marmosets were i.m. injected with HuBuChE at 16 h before the nerve agent exposure. The maximum volume injected was 400 µl divided over both legs.

II.2.14 Toxicokinetics of (±)-sarin, C(±)P(±)-soman and (±)-VX in the anesthetized, atropinized and artificially ventilated guinea pig following intravenous bolus administration

Guinea pigs (pretreated with HuBuChE) were anesthetized with a mixture (3.5 mL/kg, i.m.) of Dormicum® (midazolam, 5 mg/mL) and Hypnorm® (fentanyl, 0.315 mg/mL and fluanisone, 10 mg/mL). Cannulas were inserted into the left carotid artery and into the trachea. Heparin (60 U) was administered via the carotid cannula. The animals were mechanically ventilated using an infant ventilator (Hoek Loos, Schiedam, The Netherlands).
The jugular vein was traced and made accessible. Atropine sulfate, dissolved in sterile saline, was administered i.p. (17.4 mg/kg). A blood sample was drawn via the carotid cannula, after which a volume of sterile saline corresponding with that of the blood sample was administered through the same cannula. A dose of (±)-sarin, C(±)P(±)-soman or (±)-VX was injected into the jugular vein (injection volume 1 mL/kg). For this purpose a standard solution of sarin, soman or VX in 2-propanol was diluted with sterile saline just before use. Blood samples were drawn at various time points up to 120 min after administration of the toxicant. A corresponding volume of saline was given back after each sampling event. After taking the final blood sample the animals were sacrificed with an overdose of pentobarbital sodium (Nembutal®, 0.4 mL, i.p).
A small portion of the blood sample was used to determine the BuChE activity, whereas the larger part was used for gas chromatographic analysis. The fluoride-induced reactivation procedure was applied, on a portion of the final blood sample in order to quantify the total amount of (±)-sarin (free and bound) in the sample.

II.2.15 Toxicokinetics of (±)-sarin or C(±)P(±)- soman in the anesthetized and atropinized guinea pig during and after nose-only exposure to (±)-sarin or soman vapor in air

Toxicokinetic experiments were performed by nose-only exposure of anesthetized, atropinized and restrained guinea pigs to a concentration of (±)-sarin or C(±)P(±)-soman vapor in air which could be varied between 2 $mg.m^{-3}$ and 375 $mg.m^{-3}$. The apparatus was basically the same as developed within the context of Cooperative Agreement DAMD17-90-Z-0034 (Benschop and Van Helden. 1993; Langenberg *et al.* 1998b). During the performance of Cooperative Agreement DAMD17-94-V-4009 (Langenberg *et al.* 1998a) the exposure module was modified

in the sense that the internal volume was reduced and the pathways shortened, whereas the teflon front chamber from which the animal breathes was replaced with a stainless steel front chamber. Guinea pigs (pretreated with HuBuChE) were anesthetized with a mixture (3.5 mL/kg, i.m.) of Dormicum® (midazolam, 5 mg/mL) and Hypnorm® (fentanyl, 0.315 mg/mL and fluanisone, 10 mg/mL). A cannula was inserted into the left carotid artery. Heparin (60U) was administered via the carotid cannula. Next, the animals were atropinized by i.p. administration of 1 mL/kg of a solution of 17.4 mg of atropine sulfate per mL of saline. The animals were restrained in a modified Battelle tube (Langenberg *et al.* 1998b), with the carotid artery cannula protruding from the tube. Blood samples were taken just before starting the exposure and at various time points during or after the exposure. After taking the final blood sample the animals were sacrificed with an overdose of pentobarbital sodium (Nembutal®, 0.4 mL, i.p).
A small portion of the blood sample was used to determine the BuChE activity, whereas the larger part was used for gas chromatographic analysis. The fluoride-induced reactivation procedure was applied, on a portion of the final blood sample in order to quantify the total amount of (±)-sarin (free and bound) in the sample.

II.2.16 Toxicokinetics of soman in the anesthetized and atropinized marmoset during and after nose-only exposure to soman vapor in air

Marmosets (pretreated with HuBuChE) were anesthetized with 20 mg of Nimatek® (ketamine). Additionally, 50 µl of Vetranquil® (acepromazine, 10 mg/mL) was injected. Cannulas were inserted into the left carotid artery. Heparin (60 U) was administered via the carotid cannula. Atropine sulfate, dissolved in sterile saline, was administered i.p. (50 mg/kg). A blood sample was drawn via the carotid artery cannula, after which a volume of sterile saline corresponding with that of the blood sample was administered through the same cannula. The animals were restrained in a marmoset chair (developed within the context of DAMD17-03-1-0613), with the nose piece connected to the exposure box (see II.2.14). Blood samples were taken just before starting the 2 or 5 min exposure period and at various time points up to 60 min thereafter. After taking the final blood sample the animals were sacrificed with an overdose of pentobarbital sodium (Nembutal®, 0.4 mL, i.p).
A small portion of the blood sample was used to determine the BuChE activity, whereas the larger part was used for gas chromatographic analysis. The fluoride-induced reactivation procedure was applied, on a portion of the final blood sample in order to quantify the total amount of (±)-soman (free and bound) in the sample.

II.2.17 Toxicokinetics of VX in the anesthetized, atropinized and artificially ventilated guinea pig during and after percutaneous application of VX

Hairless guinea pigs (pretreated with HuBuChE) were anesthetized with a mixture (3.5 mL/kg, i.m.) of Dormicum® (midazolam, 5 mg/mL) and Hypnorm® (fentanyl, 0.315 mg/mL and fluanisone, 10 mg/mL). Cannulas were inserted into the left carotid artery and into the trachea. Heparin (60 U) was administered via the carotid cannula. Atropine sulfate, dissolved in sterile saline, was administered i.p. (17.4 mg/kg). The animals were mechanically ventilated using an infant ventilator (Hoek Loos, Schiedam, The Netherlands).
For percutaneous exposure a dose of (±)-VX corresponding with 2 LD50 (250 µg/kg) was pipetted on the skin of the belly of the hairless guinea pig and covered with a 2 cm^2 circular metal disk taped onto the skin immediately after application. The concentration of the (±)-VX solution in 2-propanol was 10.0 mg/mL. Therefore the pipetted volume was dependent on the weight of the animal. Blood samples were taken at various time points, up to 6 or 7 h after administration of the toxicant, after which a corresponding volume of saline was administered

via the same cannula. After taking the final blood sample the animals were sacrificed with an overdose of Nembutal® (pentobarbital).

II.2.18 Collection of guinea pig tissues

Guinea pigs (pretreated with HuBuChE) were euthanized with an overdose of Nembutal® (pentobarbital sodium, 0.4 mL i.p.). Blood was taken and treated with heparin to avoid clogging. The liver, kidney, heart, lungs and brains were removed. Homogenates (25%) in saline were prepared using a Kinematica homogenizer (PCU, Luzern, Switzerland). The homogenates were stored in the freezer at -74 °C. The BuChE activity in the tissue homogenates was measured according to the Ellman method as described in II.2.1.

II.2.19 Distribution of radioactively labeled ^{14}C-soman in HuBuChE pretreated guinea pigs after i.v. bolus administration

Guinea pigs (pretreated with HuBuChE) were anesthetized with a mixture (3.5 mL/kg, i.m.) of Dormicum® (midazolam, 5 mg/mL) and Hypnorm® (fentanyl, 0.315 mg/mL and fluanisone, 10 mg/mL). Cannulas were inserted into the left carotid artery and into the trachea. Heparin (60 U) was administered via the carotid cannula. The animals were mechanically ventilated using an infant ventilator (Hoek Loos, Schiedam, The Netherlands).
The jugular vein was traced and made accessible. Atropine sulfate, dissolved in sterile saline, was administered i.p. (17.4 mg/kg). A dose of ^{14}C-soman (55 µg, 55 mCi/mmol) was injected into the jugular vein (injection volume 1 mL). For this purpose a standard solution of ^{14}C-soman in acetonitrile was diluted with sterile saline just before use. After 90 min the animal was euthanized with an overdose of Nembutal (pentobarbital, 0.4 mL i.p.). Blood was taken and treated with heparin to avoid clogging. The liver, kidney, heart, lungs and brains were removed and 25% homogenates in saline were prepared. The homogenates were centrifuged and supernatant was removed. The residue was washed with fresh saline and again centrifuged. The supernatants were pooled. Part of the supernatant (25 µl) was mixed with 19 mL Hi-Ionic Scintillation fluid and counted in a Tri-Carb 4000 scintillation counter (Packard) for 5 min. The radioactivity in the blood sample was determined by mixing 10 µl of blood with 4.5 mL of Hi-Ionic Scintillation fluid. The homogenates and blood samples were also extracted with a mixture of toluene:butanol after acidification with sulfuric acid in order to determine the amount of hydrolyzed soman. The radioactivity in the extracts was counted after mixing with scintillation fluid. A part of the supernatants of the homogenates was loaded on a procainamide sepharose affinity column to isolate (^{14}C-soman-inhibited) BuChE (see section II.2.20).

II.2.20 Isolation of BuChE using procainamide affinity chromatography

An FPLC system from Pharmacia (Uppsala, Sweden) was utilized for the procainamide affinity chromatography to isolate BuChE from plasma or supernatants of tissue homogenate. The system consisted of two peristaltic pumps (P-1), a gradient controller (GP-250), a fraction collection module (Frac-100) and UV detector (UV-1). The column was filled with 8 mL of procainamide-Sepharose 4B gel. This gel was kindly donated by Dr. Y. Ashani, from the Israel Institute for Biological Research (Ness-Ziona, Israel), in 1992. The mobile phase of the chromatography system consisted of a gradient with buffer A (20 mM sodium phosphate, 1 mM EDTA, pH 6.9) and B (20 mM sodium phosphate, 1 mM EDTA, 600 mM sodium chloride, pH 6.9). The flow was 1 mL/min. The gradient program was:
0'-10': 100% A → 16.7% B; 10'-25': 16.7% B; 25'-35': 16.7% B → 100%B; 35'-60': 100% B; 60'-65': 100% B → 100% A; 65'-110': 100% A. Inhibited BuChE eluted from the column after 42-48 min.

II.2.21 Preparation of procainamide affinity gel

Procainamide gel was also in-house prepared. For this purpose approximately 125 mL of Sepharose 4B gel suspension in ethanol/water was placed in a funnel with a glass filter and washed with 1 liter of water, re-suspended in 0.2 M phosphate buffer (pH 11.5) and cooled at 4 °C. Cyanogen bromide (7 g in 15 mL acetonitrile/water 1:1) was added gently and the suspension was stirred for 10 min. Next the suspension was washed with water and immediately transferred into ε-aminohexanoic acid solution (1.6 g ε-aminohexanoic acid in 150 mL 0.2 M sodium carbonate, 0.2 M sodium bicarbonate and 0.4 M sodium chloride, pH 9). The mixture was stirred for 48 h at 4 °C. Next the gel was washed with water, re-suspended in water (150 mL) and after addition of 4.2 g procainamide.HCl adjusted to pH 4.5. The mixture was stirred for 60 h. Next, the gel was washed with water. The wash fluid was collected and the extinction was measured at 278 nm. It was calculated that 29 µmol of procainamide was bound to 1 mL of gel.

II.2.22 Curve-fitting of toxicokinetic data

Curve-fitting of the measured concentration-time courses was performed by nonlinear regression with TableCurve software (Jandell, AISN Software) on a personal computer equipped with an Intel Celeron 300 mHz processor, as described previously (Benschop and De Jong, 1990).
The data were fitted to a multiexponential equation :

$$[\text{nerve agent}]_t = A*e^{-at} + B*e^{-bt} + C*e^{-ct} \qquad \text{(eq. 1)}$$

by calculation of the parameters A, B, C, a, b, and c. In these equations, $[\text{nerve agent}]_t$ is the concentration of the nerve agent stereoisomer in blood under study at time t.
Several toxicokinetic parameters were calculated:

area under the curve (AUC)	$AUC = A/a + B/b + C/c$	(eq. 2)
total body clearance (Cl)	$Cl = Dose/AUC$	(eq. 3)
concentration in blood at time 0 (C_0)	$C_0 = A + B + C$	(eq. 4)
half-life of first toxicokinetic phase ($t_{½,a}$)	$t_{½,a} = \ln 2/a$	(eq. 5)
half-life of second toxicokinetic phase ($t_{½,b}$)	$t_{½,b} = \ln 2/b$	(eq. 6)
half-life of third toxicokinetic phase ($t_{½,c}$)	$t_{½,c} = \ln 2/c$	(eq. 7)
volume of the central compartment (V_1)	$V_1 = Dose/C_0$	(eq. 8)

III. RESULTS

III.1. TIME COURSE OF HuBuChE IN BLOOD OF THE GUINEA PIG FOLLOWING INTRAMUSCULAR ADMINISTRATION (T.O. 1)

In order to establish which point in time after administration of HuBuChE to guinea pigs would be optimal for a nerve agent challenge, the time course of BuChE activity was determined in blood samples drawn from three guinea pigs at various time points after administration of the enzyme, using the colorimetric method of Ellman *et al.* (1961). The results are shown in Table 1 and Figure 1.

Table 1 Activity of BuChE in blood of guinea pigs at various time points after intramuscular administration of HuBuChE.

	GP 1, 448 g			GP 2, 454 g			GP 3, 532 g		
HuBuChE dose (U/kg)	20700 (345 nmol)			20700 (345 nmol)			10500 (175 nmol)		
	Time (h)	BuChE (U/mL)	BuChE (nM)	Time (h)	BuChE (U/mL)	BuChE (nM)	Time (h)	BuChE (U/mL)	BuChE (nM)
	0	1.84	31	0	1.14	19	0	1.75	29
	1	1.92	32	1	2.20	37	1	4.54	76
	2	2.91	49	2	1.67	28	2	4.88	81
	18	30.8	514	18	33.3	554	19	21.9	365
	20	34.1	568	20	35.8	596	22	21.8	364
	22	36.3	605	22	34.0	566	26	22.9	382
	24	34.8	580	24	33.4	556	43	20.2	336
	26	38.1	636	26	34.5	575	46	21.8	364
	43	48.3	805	44.5	35.4	590	48	22.0	367
	46	53.7	895	49	29.6	493	50	21.6	361
	48	51.5	859	67	28.3	471	67	14.7	245
				73	26.0	433	71	15.6	259
							74	15.6	259

Note: 60 U corresponds with approximately 1 nmol

As expected, the baseline activities of BuChE in the blood of the three guinea pigs shows some variation. After i.m. administration of HuBuChE the BuChE activity in blood increases with a half-life of approx. 5 h (which largely corresponds with the half-life of absorption) up to 20-24 h after administration, after which the activity remains relatively constant for another 24 h. Subsequently, the activity declines with a half-life of *ca.* 60 h (which is largely the elimination half-life). In the studied dose range, the maximum BuChE activity that is reached appears to be linear with the dose.

We wish to emphasize here that we were not able to obtain adequate fits to the pharmacokinetic equation: $BuChE\ activity = A*exp(-k_{el}*t)-B*exp(-k_{abs}*t)+C$, in which k_{abs} is the absorption rate constant, k_{el} the elimination rate constant, and C the BuChE activity at time zero. This is mainly due to an insufficient number of data in the elimination phase of the curve.

In guinea pig #1 the pattern seems to be aberrant, as the BuChE activity increases considerably after leveling off around 20-24 h, for which we have no explanation at this point. It seems that this guinea pig received a second dose of HuBuChE, which however was not the case.

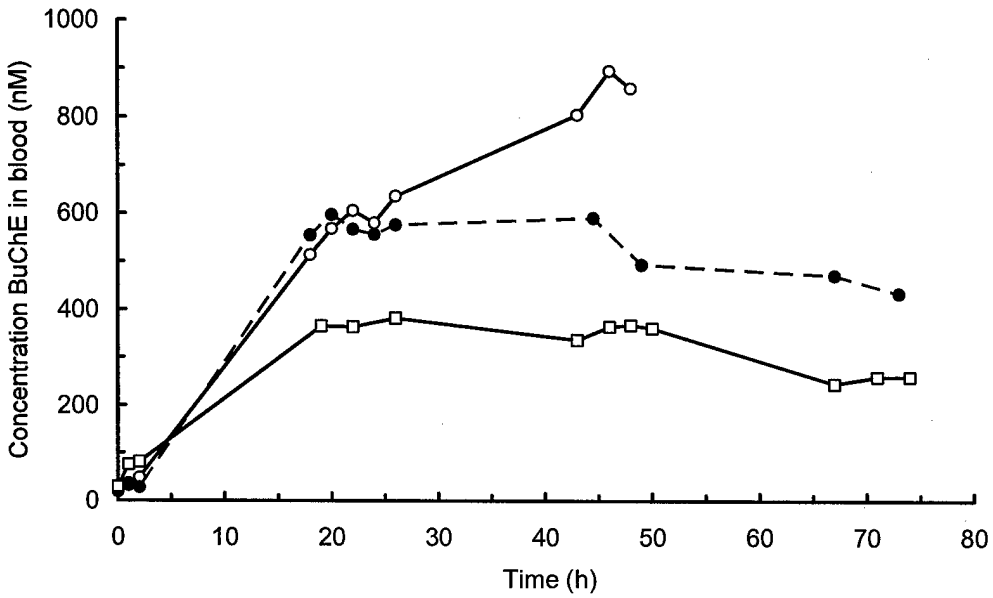

Figure 1 Concentration-time courses of BuChE-concentration measured in blood of guinea pigs # 1 (○), 2 (●) and 3 (□), following intramuscular administration of HuBuChE.

III.2 TOXICOKINETICS OF C(±)P(±)-SOMAN STEREOISOMERS IN ANESTHETIZED, ATROPINIZED AND ARTIFICIALLY VENTILATED GUINEA PIGS PRETREATED WITH HuBuChE AFTER INTRAVENOUS BOLUS ADMINISTRATION (T.O.s 2 AND 3)

III.2.1 Gas chromatographic analysis of soman stereoiosomers

The gas chromatographic analysis of soman was performed on a new instrument (see section II.2.7). Samples were loaded on Tenax tubes, then thermally desorbed and trapped in a cooled injection system. The soman components were separated from the matrix components on the precolumn. Next the components were focused in a cold trap and injected onto the chiral column to obtain chiral resolution of the soman isomers. Figure 2 shows a typical separation of the soman isomers using the 2D-chromatography system. The detection limit of the soman isomers was about 5 pg for each isomer.

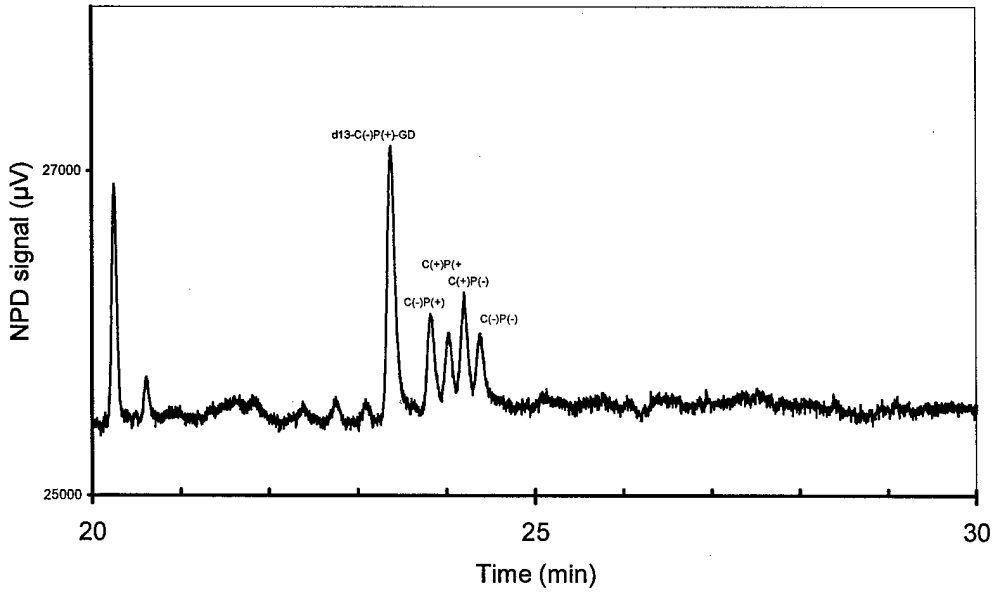

Figure 2 Typical separation of the stereoisomers of soman and internal standard d13-C(-)P(+)-soman using the 2D-GC configuration

The lowest concentration of soman in blood that could be detected was about 50 pg/mL. This relatively high detection limit is determined by the size of the blood samples, the efficacy of the solid phase extraction and the volume of the eluate. Suppose a blood sample of 500 µl with a concentration of 50 pg/mL. This sample will contain only 25 pg of soman. The efficacy of the solid phase extraction is estimated to be 80 % and the sample is recovered in 1 mL ethyl acetate. Only a maximum of 400 µl sample is loaded on the Tenax tube, which means that only 40% of the amount of soman is injected onto the chromatography column. Combining these efficiencies the amount of soman that is injected onto the column in this particulate sample is 25 pg * 80% * 40% = 8 pg, which is in reasonable agreement with the detection limit. In earlier studies the lowest detectable concentration of soman was lower *i.e.* down to 10 pg/mL. In those studies it was allowed to take larger blood samples. Secondly, more animals were used to construct one toxicokinetic curve. In this study the limiting factor was the availability of the HuBuChE enzyme, which means that the amount of animals that could be pretreated with the enzyme was limited as well. A consequence of the limiting amount of animals is that the maximum amount of blood that can be sampled for one toxicokinetic curve is limited as well.

III.2.2 Toxicokinetics of C(±)P(±)-soman in blood of the guinea pig after intravenous administration of a dose corresponding with 2 LD50 (55 µg/kg)

The toxicokinetics of soman stereoisomers in anesthetized, atropinized and artificially ventilated guinea pigs at an intravenous dose of C(±)P(±)-soman corresponding to 2 LD50 were investigated. Blood samples of 0.2 mL were drawn at t= 0, 1, 2 and 4 min, whereas a 0.5 mL blood sample was drawn after 10 min and 1.0 mL blood samples were drawn at 20, 40 and 60 min after injection of the toxicant. Although the toxicokinetics of soman in guinea pigs have been measured before (Benschop and de Jong, 1991), the toxicokinetics were also measured in animals that were not pretreated with HuBuChE in order to obtain adequate reference curves. The P(+)-isomers of soman were not detected in any of the blood samples. The concentrations of the P(-) isomers of soman in blood are presented in Table 2. The data were fitted in a two-exponential curve which is shown in Figure 3. The toxicokinetic parameters can be found in Table 3.

The fluoride reactivation technique was applied to the final blood sample that was drawn in each toxicokinetic experiment. Only the regenerable soman molecules can be measured. Blood of the guinea pig contains significant amounts of CaE which can be regenerated with fluoride ions. The value provides an additional positive verification of the exposure to the toxicant.

Table 2 Concentration (ng/mL) of C(+)P(-)- and C(-)P(-)-soman in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg).

Animal #	GP 1, 440 g		GP 2, 463 g		Mean ± SEM	
	C(+)P(-)-	C(-)P(-)-	C(+)P(-)-	C(-)P(-)-	C(+)P(-)-	C(-)P(-)-
Time (min)	Soman	Soman	Soman	Soman	Soman	Soman
1	1.62	7.07	1.28	6.16	1.45 ± 0.2	6.61 ± 0.5
2	0.56	2.61	0.87	3.16	0.71 ± 0.2	2.89 ± 0.3
4	0.40	1.48	0.12	2.02	0.41 ± 0.0	1.75 ± 0.3
10	0.15	0.50	0.17	0.57	0.16 ± 0.01	0.54 ± 0.03
20	0.09	0.16	< LOD	0.18	0.096	0.17 ± 0.01
40	< LOD	0.061	< LOD	< LOD		0.06
60	< LOD		< LOD	< LOD		
F-react. *		57.2		57.8		57.5

LOD = limit of detection (50 pg/mL), *F- react. is expressed in ng regenerated C(±)P(±)-soman / mL plasma

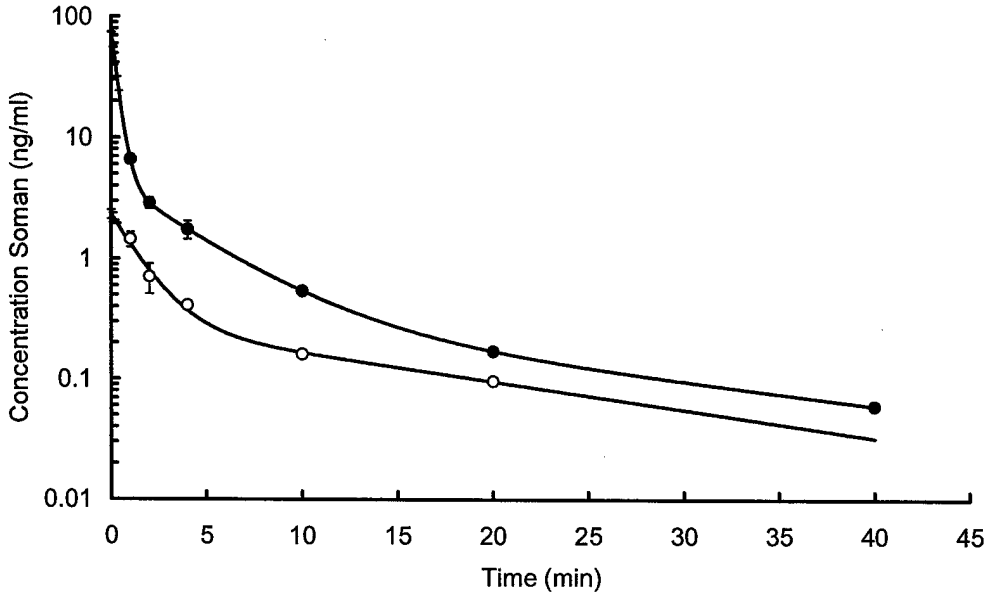

Figure 3 Mean concentration (ng/mL) ± SEM of C(+)P(-)-soman (○) and C(-)P(-)-soman (●) in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg).

Table 3 Toxicokinetic parameters for C(±)P(-)-soman in anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of C(±)P(±)-soman a dose corresponding with 2 LD50 (55 µg/kg).

Parameter	Dimension	C(+)P(-)-soman	C(-)P(-)-soman
Dose	$\mu g.kg^{-1}$	15.1	12.3
Number of exponents		2	3
A	$ng.mL^{-1}$	2.05	70.0
B	$ng.mL^{-1}$	0.658	4.05
C	$ng.mL^{-1}$	-	0.356
a	min^{-1}	0.277	3.11
b	min^{-1}	0.0536	0.256
c	min^{-1}	-	0.0445
AUC	$ng.min.mL^{-1}$	8.81	46.3
C0	$ng.mL^{-1}$	2.29	74.4
t½,a	min	1.2	0.22
t½,b	min	14.7	2.7
t½,c	min	-	15.5
Vl	$l.kg^{-1}$	6.6	0.166
Cl	$l.min.kg^{-1}$	1.72	0.268

In the next series of experiments, animals were pretreated with HuBuChE. The dose of HuBuChE was established at 0.7 * dose of soman which corresponds with 0.7 *55/182 = 211 nmol HuBuChE /kg. Soman was intravenously injected at 24 h after the i.m. injection of HuBuChE. The levels of HuBuChE in blood at 24 after i.m. injection of HuBuChE were approximately the same as found in section III.1. Only C(-)P(-)-soman was detected in the blood

samples. The deviation in the measured concentrations of C(-)P(-)-soman in blood in the various animals was rather high. Table 4 shows two columns with averaged concentrations. The forelast shows the average values of all animal experiments. The last shows the average levels of all animals except those for animal 3. The soman levels in blood of animal 3 were rather high combined with a rather low BuChE concentration in the corresponding blood samples. The levels in the other animals were much lower indicating a significant effect of the scavenger. The BuChE levels in blood were also rather persistent compared to the levels in the blood of animal 3. The levels of BuChE were higher than baseline levels normally found in guinea pigs, which are approximately 20 nM. The average curve of the soman levels in blood was constructed according to the data shown in the forelast column. The data were fitted to a bi-exponential curve which is shown in Figure 4. The toxicokinetic parameters are shown in Table 5.

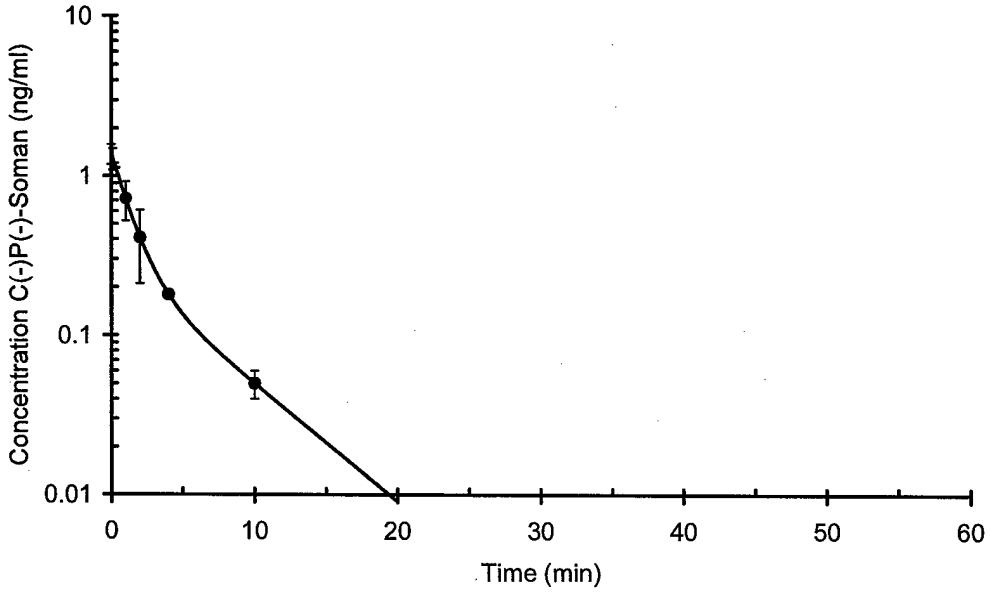

Figure 4 Mean concentration ± SEM (ng/mL) of C(-)P(-)-soman (•) in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs that were pretreated with HuBuChE (200 nmol/kg) after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg).

Table 4 Concentrations of C(+)P(-)- and C(-)P(-)-soman (ng/ml) concentration of BuChE (nM) in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg). At 24 h before C(±)P(±)-soman administration guinea pigs were injected (i.m.) with HuBuChE (100 nmol/500g ≡ 6000 U).

Animal	GP 3, 530g			GP 4, 472 g			GP 5, 459 g			GP 6, 411 g		
Time (min)	C(+)P(-)-Soman	C(-)P(-)-Soman	BuChE (nM)	C(+)P(-)-Soman	C(-)P(-)-Soman	BuChE (nM)	C(+)P(-)-Soman	C(-)P(-)-Soman	BuChE (nM)	C(+)P(-)-Soman	C(-)P(-)-Soman	BuChE (nM)
0	<LOD	< LOD	665	< LOD	< LOD	709	< LOD	< LOD	695	< LOD	< LOD	643
1	<LOD	5.59	97	< LOD	0.30	179	< LOD	0.50	150	< LOD	0.66	67
2	< LOD	0.895	26	< LOD	< LOD	148	< LOD	0.29	91	< LOD	0.34	63
4	< LOD	0.324	30	< LOD	< LOD	67	< LOD	0.19	52	< LOD	0.10	60
10	< LOD	0.101	23	< LOD	< LOD	115	< LOD	0.054	34	< LOD	0.054	39
20	< LOD	0.03	21	< LOD	< LOD	99	< LOD	< LOD	89	< LOD	< LOD	36
40	< LOD	< LOD	27	< LOD	< LOD	125	< LOD	< LOD	51	< LOD	< LOD	37
60	< LOD	< LOD	27	< LOD	< LOD	127	< LOD	< LOD	36	< LOD	< LOD	31
F-react		65			51.2						67	

LOD = limit of detection (50 pg/mL), F- react expressed in ng regenerated soman per mL plasma.

Table 4 (Continued) Concentrations of C(+)P(-)- and C(-)P(-)-soman (ng/ml) concentration of BuChE (nM) in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg). At 24 h before C(±)P(±)-soman administration guinea pigs were injected (i.m.) with HuBuChE (100 nmol/500g ≡ 6000 U).

Animal	GP 7, 610 g			GP 8, 547 g			Mean ± SEM[1]			Mean ± SEM[2]		
Time (min)	C(+)P(-)-Soman	C(-)P(-)-Soman	BuChE (nM)	C(+)P(-)-Soman	C(-)P(-)-Soman	BuChE (nM)	C(+)P(-)-Soman	C(-)P(-)-Soman	BuChE (nM)	C(+)P(-)-Soman	C(-)P(-)-Soman	BuChE (nM)
0	< LOD	<LOD	596	< LOD	< LOD	829	< LOD	< LOD	690± 79	< LOD	< LOD	694 ± 39
1	< LOD	1.43	55	< LOD	0.72	153	< LOD	1.53 ± 2.0	117± 51	< LOD	0.72 ± 0.2	121 ± 25
2	< LOD	0.75	68	< LOD	0.26	118	< LOD	0.51 ± 0.3	86 ± 43	< LOD	0.41 ± 0.1	98 ± 16
4	< LOD	0.29	65	< LOD	0.13	140	< LOD	0.21 ± 0.1	69 ± 37	< LOD	0.18 ± 0.04	77 ± 16
10	< LOD	0.05	46	< LOD	0.03	93	< LOD	0.06 ± 0.03	58 ± 37	< LOD	0.05 ± 0.01	65 ± 16
20	< LOD	< LOD	56	< LOD	< LOD	101	< LOD	0.03 ± 0.01	67 ± 34	< LOD	< LOD	76 ± 13
40	< LOD	< LOD	49	< LOD	< LOD	86	< LOD	< LOD	63 ± 36	< LOD	< LOD	70 ± 16
60	< LOD	< LOD	63	< LOD	< LOD	99	< LOD	< LOD	64 ± 41	< LOD	< LOD	71 ± 18
F-react		63			63			62 ± 6			62 ± 3	

LOD = limit of detection (50 pg/mL), F- react expressed in ng regenerated soman per mL plasma, 1) all results, 2) results excluding animal 3

Table 5 Toxicokinetic parameters for C(+)P(-)-and C(-)P(-)-soman in anesthetized, atropinized and mechanically ventilated guinea pigs that were pretreated with HuBuChE (200 nmol/kg) after i.v. bolus administration of C(±)P(±)-soman at a dose corresponding with 2 LD50 (55 µg/kg).

Parameter	Dimension	C(+)P(-)-soman	C(-)P(-)-soman
Dose	$\mu g.kg^{-1}$	15.1	12.4
Number of exponents			
A	$ng.mL^{-1}$	-	1.11
B	$ng.mL^{-1}$	-	0.274
C	$ng.mL^{-1}$		-
a	min^{-1}	-	0.815
b	min^{-1}	-	0.179
c	min^{-1}		-
AUC	$ng.min.mL^{-1}$	0	2.89
C0	$ng.mL^{-1}$	0	1.38
t½,a	min	-	0.85
t½,b	min	-	3.87
t½,c	min	-	-
Vl	$l.kg^{-1}$	-	8.95
Cl	$l.min.kg^{-1}$	-	4.28

In another series of experiments (see T.O. 3) guinea pigs were challenged with multiple LD50's of soman. Guinea pigs were pretreated with HuBuChE (210 nmol/kg) and at 24 h after the enzyme injection the animals were operated to install cannula in the trachea and carotid artery. The animals were artificially respirated and atropinized. Next, a dose of soman corresponding with 2 LD50 was intravenously injected. Blood samples were not taken. At 90 min after the first challenge with soman, a second injection of soman corresponding with 2 LD50 was given. After this challenge blood samples were taken, processed and analyzed with 2D-GC. The blood sample size was 0.25 mL for time points 0, 1, 2 and 4 min. Blood sample volumes were 0.5 mL for the time points 10, 20, 40, 60 and 2 mL for the final blood sample taken at 120 min after soman injection. The results are shown in Table 6. Both C(±)P(-)-isomers of soman could be detected in the blood samples. The levels of soman in blood were also higher than the levels found in the non-pretreated animals, indicating that non-pretreated animals had more binding sites available than the pretreated animals that were pre-intoxicated with an extra dose of soman. The C(-)P(-)-soman isomer concentration curve was fitted to a three exponentials equation whereas the C(+)P(-)-soman isomer concentration curve was fitted to a bi-exponential equation. Figure 5 shows the curves. Table 7 lists the toxicokinetic parameters.

Table 6 Concentrations of C(+)P(-)- and C(-)P(-)-soman (ng/ml) and BuChE (nM) in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg) and a 2nd dose of soman corresponding with 2LD50 (55 µg/kg) given at 90 min after the first dose. At 24 h before the first injection with soman, guinea pigs were injected (i.m.) with HuBuChE (100 nmol/500g ≡ 6000 U).

Animal #	GP 1, 450 g			GP 2, 478 g			GP 3, 487 g		
Time (min)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)
-90	<LOD	<LOD	480	<LOD	<LOD	884	<LOD	<LOD	938
0	<LOD	<LOD	6	<LOD	<LOD	204	<LOD	<LOD	61
1	14	16.1	-6	0.25	5.26	10	n.m.	n.m.	n.m.
2	7.15	8.49	8	0.16	1.86	-50	0.09	0.29	9
4	3.55	4.52	8	0.11	0.80	27	0.09	0.36	10
10	0.90	1.61	2	0.068	0.20	9	0.14	0.49	3
20	0.21	0.454	3	<LOD	0.047	25	0.12	0.15	3
40	< LOD	0.080	1	<LOD	<LOD	14	<LOD	0.038	-30
60	< LOD	0.030		<LOD	<LOD	12	<LOD	<LOD	10
F-react		97			59			57	

LOD = limit of detection (50 pg/mL) , n.m. = mot measured, F- react expressed in ng regenerated soman per mL plasma

Table 6 (Continued) Concentrations of C(+)P(-)- and C(-)P(-)-soman (ng/ml) and BuChE (nM) in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg) and a 2nd dose of soman corresponding with 2LD50 (55 µg/kg) given at 90 min after the first dose. At 24 h before the first injection with soman, guinea pigs were injected (i.m.) with HuBuChE (100 nmol/500g ≡ 6000 U).

Animal	GP 4, 575 g			GP 5, 653 g			GP 6, 680 g			Mean		
Time (min)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)
-90	<LOD	<LOD	1118	<LOD	<LOD	763	<LOD	<LOD	1101	<LOD	<LOD	880 ± 97
0	<LOD	<LOD	215	<LOD	<LOD	77	<LOD	<LOD	102	<LOD	<LOD	110 ± 34
1	27.8	43	-1	13	17.5	5.8	13.9	20.0	7	17.2 ± 3	24.0 ± 2.5	3 ± 2.6
2	10.0	16	-7	6.5	8.03	5.3	7.72	11.2	1	6.3 ± 2	8.74 ± 2	5 ± 9
4	3.8	7.4	-8	3.3	4.5	6.1	4.35	6.78	10	3.0 ± 0.7	4.70 ± 0.7	8 ± 5
10	0.80	2.7	22	0.89	1.8	2.4	1.06	2.64	2	0.75 ± 0.2	1.83± 0.3	6 ± 3
20	0.11	0.82	4	0.18	0.58	3.8	0.22	0.73	7	0.17 ± 0.02	0.55 ± 0.1	5 ± 4
40	<LOD	0.16	-15	0.02	0.11	6.8	<LOD	0.11	10	0.02	0.10 ± 0.02	2 ± 6
60	<LOD	<LOD	20	<LOD	<LOD	2.8	<LOD	0.058	15	<LOD	0.04 ± 0.01	1 ± 6
F-react		136			106			130			98± 18	

LOD = limit of detection (50 pg/mL), n.m. = mot measured, F- react expressed in ng regenerated soman per mL plasma

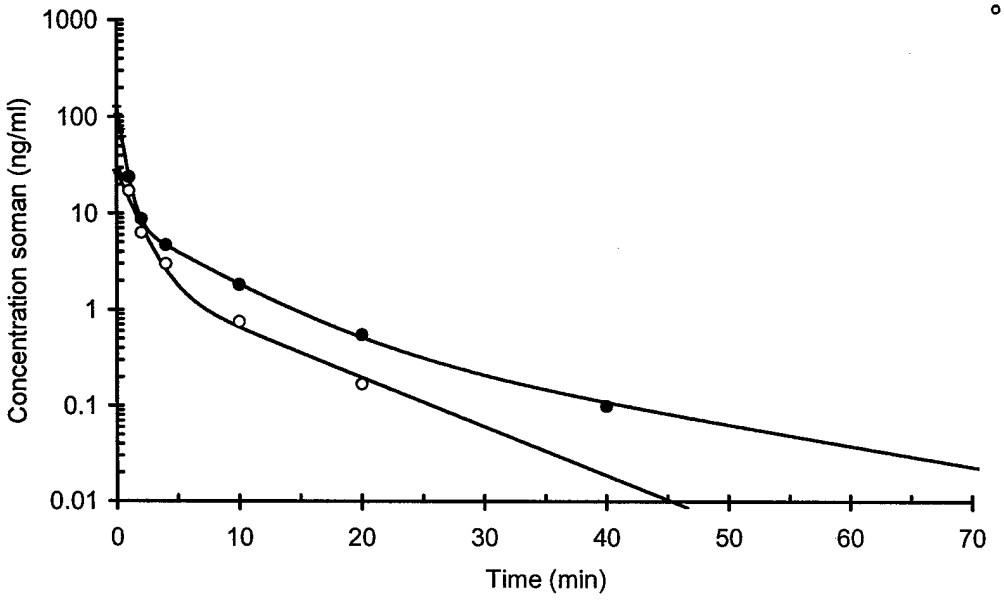

Figure 5 Mean concentration (ng/mL) of C(+)P(-)-soman (○) and C(-)P(-)-soman (●) in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs that were pretreated with HuBuChE (200 nmol/kg) after i.v. bolus administration of two doses of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg) at t= -90 and t = 0 min.

The BuChE concentration in blood decreased to negligible levels after injection of the 2nd dose of soman. Figure 6 shows the combined result of the BuChE levels after two injections of soman.

The final blood samples were used for the fluoride reactivation assay. The last line in Table 6 shows that the amount of regenerable soman was higher than the levels in the animals that received only one dose of soman, which is an indication that not all regenerable binding sites were occupied after one injection of soman. It must be noted that BuChE is not a regenerable binding site for soman with fluoride ions.

Table 7 Toxicokinetic parameters for C(+)P(-)- and C(-)P(-)-soman in anesthetized, atropinized and mechanically ventilated guinea pigs that were pretreated with HuBuChE (200 nmol/kg) after i.v. bolus administration of a C(±)P(±)-soman dose corresponding with 2 LD50 (55 µg/kg) and an additional dose of 2LD50 soman given 90 min after the first dose

Parameter	Dimension	C(+)P(-)-soman	C(-)P(-)-soman
Dose	$\mu g.kg^{-1}$	15.1 + 15.1 (after 90')	12.3 + 12.3 (after 90')
Number of exponents		2	3
A	$ng.mL^{-1}$	20.0	119
B	$ng.mL^{-1}$	0.319	8.14
C	$ng.mL^{-1}$	-	0.75
a	min^{-1}	0.455	1.96
b	min^{-1}	0.0316	0.176
c	min^{-1}	-	0.0498
AUC	$ng.min.mL^{-1}$	54.1	121.8
C0	$ng.mL^{-1}$	20.3	128
t½,a	min	1.52	0.35
t½,b	min	21.9	3.94
t½,c	min	-	13.9
Vl	$l.kg^{-1}$	1.49	0.193
Cl	$l.min.kg^{-1}$	0.559	0.203

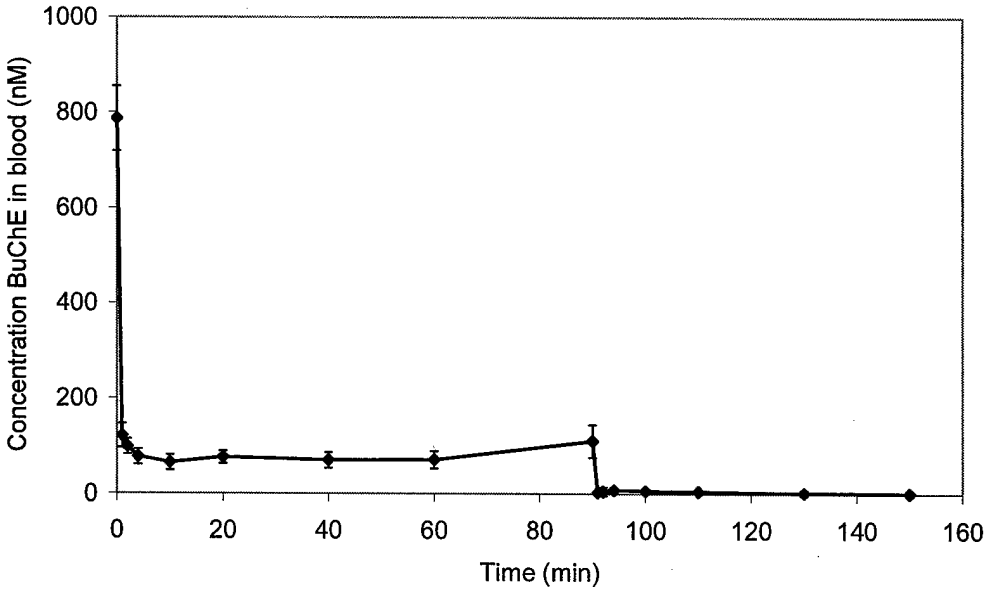

Figure 6 Concentration (nM) of BuChE in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs that were pretreated with HuBuChE (200 nmol/kg) after i.v. bolus administration of two doses dose of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg) at t= -90 and t = 0 min.

III.3 TOXICOKINETICS OF THE STEREOISOMERS OF (±)-SARIN IN ANESTHETIZED, ATROPINIZED AND ARTIFICIALLY VENTILATED GUINEA PIGS THAT WERE PRETREATED WITH HuBuChE AFTER INTRAVENOUS BOLUS ADMINISTRATION (T.O. 4)

III.3.1. Gas chromatographic analysis of the stereoisomers of (±)-sarin

Initially the two-dimensional gas chromatography system was configured with a flow-controlled TCT injection system. Recently a new, pressure-regulated, TCT-injector became available, which we intended to use in this study. Unfortunately, we did not succeed in getting a reproducible injection with this set-up, most likely due to interference of the pressure-controlled TCT injection and the back-pressure from the MUSIC-system. Consequently, we reverted to the old, proven, configuration for trace analysis of sarin stereoisomers (Spruit *et al*, 2001). Furthermore, we encountered unexpected difficulties in finding Cyclodex columns that provided sufficient resolution between the sarin stereoisomers. According to the manufacturer all columns have the same specifications as verified with a test mixture. Apparently, the chiral resolution of (±)-sarin is more critical. We observed that around 70 °C the cyclodextrin phase underwent a phase transfer between solid and liquid. In order to obtain resolution between sarin stereoisomers, a liquid phase is needed. However, by increasing the temperature in order to ensure the cyclodextrin phase to be liquid, we had insufficient chromatographic resolution. The manufacturer gave us the opportunity to test a number of columns with (±)-sarin on our configuration, after which we were able to select a few specimens that met with our criteria. The detection limit of sarin was 5 pg per isomer (S/N=3). Based on the injection volume of 400 µl, concentrations down to 40 pg/mL could be determined with statistical significance (S/N=10). Analysis of blank blood samples showed no peaks with the same retention times as (+)-sarin, (-)-sarin or the internal standard (-)-d7-sarin.

III.3.2 Toxicokinetics of (±)-sarin in blood of the guinea pig after intravenous administration of a dose corresponding with 2 LD50

Thusfar, the intravenous toxicokinetics of (±)-sarin have not been studied as extensively as those of C(±)P(±)-soman. In fact, only a dose corresponding with 0.8 LD50 (19.2 µg/kg) has been studied (Benschop and Van Helden, 1993; Spruit *et al.*, 2000). When we proposed this study it was our intention to compare the toxicokinetics for 2 LD50 in HuBuChE-pretreated guinea pigs with those for 0.8 LD50 in non-pretreated animals, assuming a dramatic effect on the toxicokinetics of pretreatment with HuBuChE. As will become apparent, the effect of the scavenger on the toxicokinetics is not that dramatic. As a consequence we decided to perform a limited study on the toxicokinetics of sarin at a dose corresponding with 2 LD50 in naive animals.

Sarin stereoisomers were measured in the blood of anesthetized, atropinized and artificially ventilated guinea pig after i.v. administration of a dose of (±)-sarin corresponding with 2 LD50, i.e. 48 µg/kg. Blood samples were drawn at time 0 (just before administration of (±)-sarin) and at 1, 2, 4, 10, 20, 40, 60 and 120 min after the bolus injection. The drawn blood volumes ranged from 0.3 up to 2 mL (for the final sample).

(+)-Sarin was not detected in any of the blood samples (< 40 pg/mL blood). The measured concentrations of (-)-sarin in the naive animals are listed in Table 8. The mean concentration-time course is shown in Figure 7.

Up to 20 min after the i.v. bolus injection the concentration of (-)-sarin in blood decreases as expected. Oddly enough, after that the concentration remains stable, which means that under these conditions (-)-sarin is far more persistent *in vivo* than anticipated. This result is in agreement with the findings published by Spruit *et al* (2000).

Table 8 Concentration (ng/mL) of (-)-sarin in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a dose of (±)-sarin corresponding with 2 LD50 (48 µg/kg).

Animal #	GP 1, 685 g	GP 2, 574 g	GP 3, 685 g	GP 4, 640 g	Mean ± SEM
Time (min)	[(-)-sarin]	[(-)-sarin]	[(-)-sarin]	[(-)-sarin]	[(-)-sarin]
1	8.18	4.24	24.0	6.96	10.8 ± 4.4
2	3.64	2.40	8.16	2.86	4.27 ± 1.3
4	1.00	1.00	1.69	0.77	1.12 ± 0.19
10	0.20	0.15	0.29	0.15	0.19 ± 0.03
20	0.10	0.11	0.20	0.13	0.13 ± 0.02
40	0.11	0.14	0.17	0.13	0.14 ± 0.01
60	0.10	0.12	0.30	0.15	0.17 ± 0.05
120	<LOD	0.11	<LOD	0.14	0.12 ± 0.01
F-react	56	90	44	22	53 ± 14

LOD = limit of detection (40 pg/mL), F-react is expressed in ng regenerable sarin per mL plasma

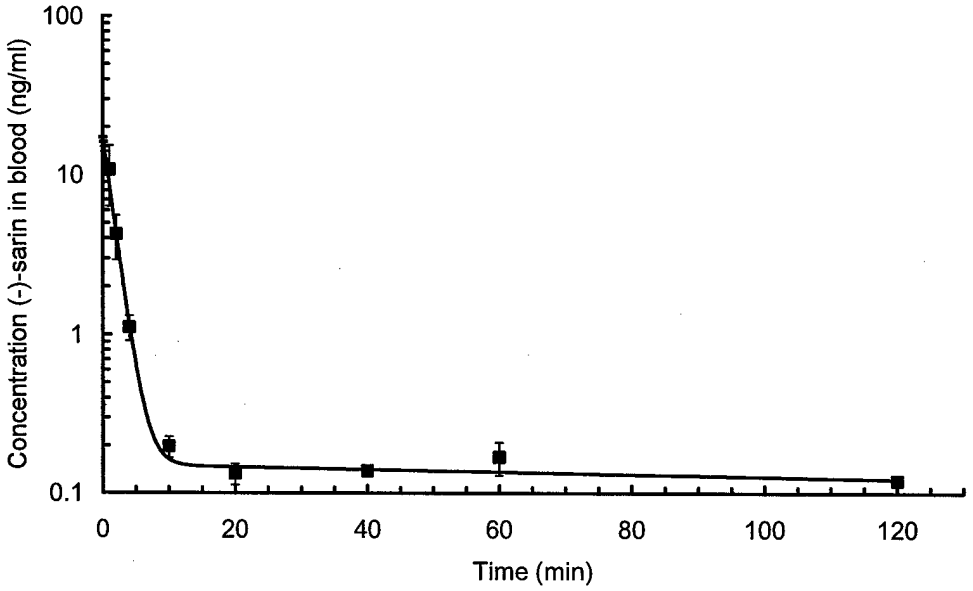

Figure 7 Mean concentration-time course of (-)-sarin (ng/mL ± SEM, n=4) in blood of anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. administration of (±)-sarin at a dose of 48 µg/kg, which corresponds with 2 LD50.

The data in Table 8 were fitted to a bi-exponential equation: $A.e^{-at}+B.e^{-bt}$. The toxicokinetic parameters derived from this fit are presented in Table 9.

Table 9 Toxicokinetic parameters for (-)-sarin in anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a (±)-sarin dose corresponding with 2 LD50 (48 µg/kg).

Parameter	Dimension	Value
Dose	$\mu g.kg^{-1}$	24
Number of exponents		2
A	$ng.mL^{-1}$	17.1
B	$ng.mL^{-1}$	0.151
a	min^{-1}	0.705
b	min^{-1}	0.00178
AUC	$ng.min.mL^{-1}$	109
AUC(1-60')	$ng.min.mL^{-1}$	20.4
C0	$ng.mL^{-1}$	17.3
t½,a	min	0.98
t½,b	min	389
Vl	$l.kg^{-1}$	1.38
Cl	$l.min.kg^{-1}$	0.22

III.3.3 Toxicokinetics of (±)-sarin in blood of the HuBuChE-pretreated guinea pig after intravenous administration of a dose corresponding with 2 LD50

In a subsequent series of experiments, guinea pigs were pretreated with HuBuChE. At 24 h before administration of the toxicant, guinea pigs were injected with a dose of HuBuChE corresponding with 0.7 times the molar dose of 2 LD50 C(±)P(±)-soman (i.v.). The i.v. LD50 of C(±)P(±)-soman is 27.5 µg/kg (Benschop and De Jong, 1991), which corresponds with 0.15 µmol/kg. The dose of HuBuChE required for pretreatment therefore is 2*0.7*0.15=0.21 µmol HuBuChE/kg. Table 10 shows the amounts of HuBuChE that were actually injected into the individual animals, which deviated from the required amounts to some extent. The reason for the this the lack of reproducibility of the recovery of HuBuChE from the glycerol preparation. Furthermore, Table 10 shows also concentrations of (-)-sarin measured in the various blood samples. These data are also represented in Figures 8 and 9. The (+)-isomer could not be detected in any of the blood samples.

Upon comparison of the data in Tables 8 and 10 it appears that generally the concentrations of (-)-sarin are lower in the HuBuChE-pretreated animals than in the naive animals. The difference is most prominent in the first few minutes after administration of (±)-sarin, but is also observable in the terminal phase of the curves.

In experiments GP 3 and 5, and presumably 2, (-)-sarin appears to be captured by the scavenger almost immediately. This is what we anticipated for the toxicokinetic curves to look like in the presence of HuBuChE.

Furthermore it is obvious from the data on BuChE in Table 10, that there is quite a bit of active BuChE left at the end of the toxicokinetic experiment, whilst (-)-sarin is still circulating at low (but still easily detectable) concentrations.

Unfortunately, the variation in the results for (-)-sarin is such that the individual values should not be averaged in order to obtain a mean concentration-time course. The curves of guinea pig 1 and 4 were averaged and a biexponential curve was fitted through these data points (see Figure 8). The concentrations in blood of guinea pig 2, 3 and 5 are plotted in Figure 9. It was not possible to construct an exponential curve through these points. The AUC was derived by numerical integration.

Table 10 Concentrations of (-)-sarin (ng/mL) and BuChE (nM) in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. administration of a dose of (±)-sarin corresponding with 2 LD50 (48 µg/kg). Guinea pigs were injected (i.m.) with HuBuChE 24 h before (±)-sarin administration.

	GP 1, 535 g		GP 2, 695 g		GP 3, 640 g		GP 4, 685 g		GP 5, 790 g	
HuBuChE dose (nmol)	86		133		145		108		136	
Time (min)	(-)-sarin	BuChE	(-)-sarin	BuChE	(-)-sarin	BuChE	(-)-sarin	BuChE	(-)-sarin	BuChE
0	0	625	0	875	0	1067	0	457	0	609
1	2.03	125	x	370	0.11	262	3.51	70	0.18	166
2	1.49	100	0.11	465	0.13	221	2.59	20	0.19	161
4	1.20	66	x	311	0.19	210	0.95	14	0.17	143
10	0.13	70	0.11	256	0.37	240	0.18	4	0.14	115
20	0.10	108	0.08	225	0.20	220	0.15	19	0.22	158
40	0.063	66	0.08	193	0.15	260	0.10	33	x	155
60	0.070	90	0.07	193	0.09	256	0.09	21	x	168
120	0.160	90	0.05	211	0.05	230	n.s.	n.s.	n.s.	n.s.
F-react.	n.s.		n.s.		344		388		405	

x= analysis failed, n.s.= not sampled, F-react is expressed as ng reactivated sarin per ml plasma

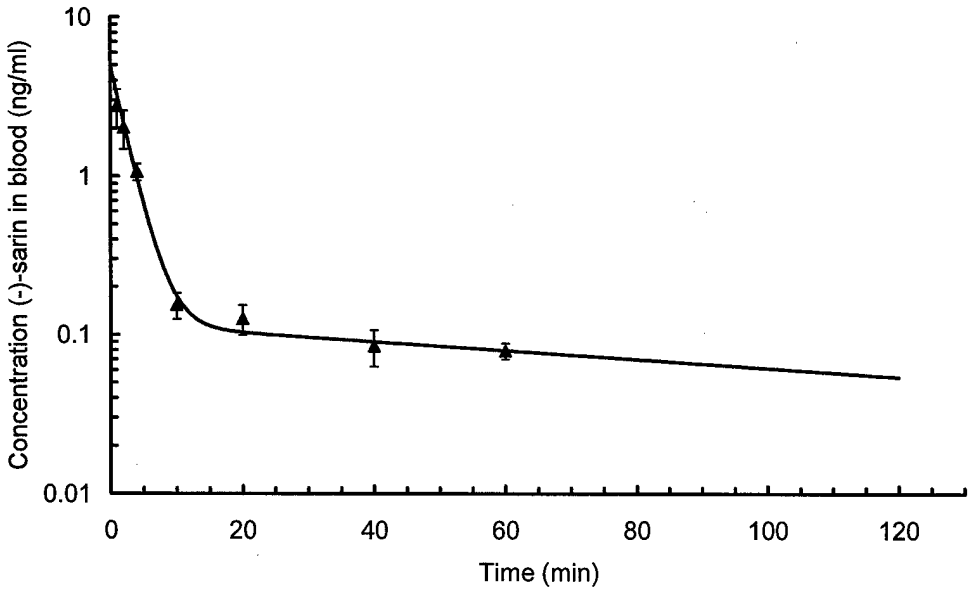

Figure 8 Mean concentration-time courses of (-)-sarin (ng/mL) in blood of anesthetized, atropinized, mechanically ventilated and HuBuChE-pretreated guinea pigs after i.v. administration of a (±)-sarin dose of 48 µg/kg, which corresponds with 2 LD50. Mean values of guinea pig GP 1 and guinea pig GP 4.

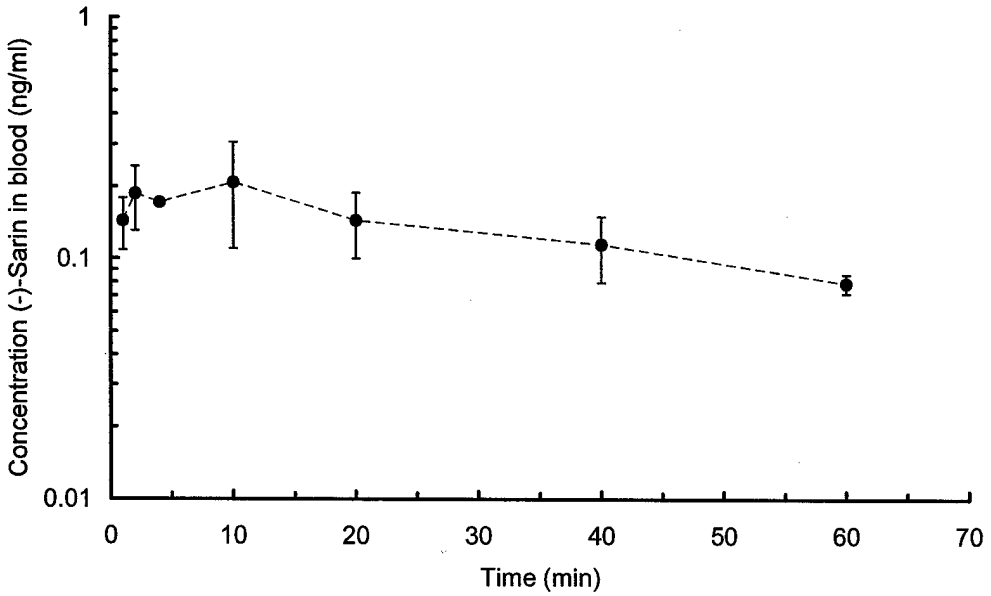

Figure 9 Mean concentration-time courses of (-)-sarin (ng/mL ± SEM, n=3) in blood of anesthetized, atropinized, mechanically ventilated and HuBuChE-pretreated guinea pigs after i.v. administration of a (±)-sarin dose of 48 µg/kg, which corresponds with 2 LD50. Mean values of guinea pig GP 2,guinea pig GP 3 and guinea pig GP 5.

The blood samples taken at the final time point were also subjected to our fluoride-induced reactivation method (Polhuijs *et al.*, 1997). Incubation with a high concentration of fluoride ions under acidic conditions releases the phosphyl moiety covalently bound to BuChE and other binding sites such as CaE, thus (re)generating (±)-sarin that can be measured gas chromatographically. The results are presented in the last line of Table 10.

Table 11 Toxicokinetic parameters for (-)-sarin in anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a (±)-sarin dose corresponding with 2 LD50 (48 µg/kg).

Parameter	Dimension	GP 1 and 4	GP 2, 3 and 5
Dose	$\mu g.kg^{-1}$	24	24
Number of exponents		2	
A	$ng.mL^{-1}$	4.80	
B	$ng.mL^{-1}$	0.116	
a	min^{-1}	0.428	
b	min^{-1}	0.00637	
AUC	$ng.min.mL^{-1}$	11.2	
AUC(1-60')	$ng.min.mL^{-1}$	13.0	2.4
C0	$ng.mL^{-1}$	5.22	
t½,a	min	5.97	
t½,b	min	108	
Vl	$l.kg^{-1}$	4.59	
Cl	$l.min.kg^{-1}$	2.14	

III.4 TOXICOKINETICS OF (±)-VX STEREOISOMERS IN ANESTHETIZED, ATROPINIZED AND ARTIFICIALLY VENTILATED HAIRLESS GUINEA PIGS PRETREATED WITH HuBuChE AFTER INTRAVENOUS BOLUS ADMINISTRATION (T.O. 5)

III.4.1 Gas chromatographic analysis of VX

The analysis of VX was performed according to the same method as described earlier in the final report of DAMD 17-97-2-7001 and published by Van der Schans *et al* (2003). Blood samples were extracted with a mixture of n-hexane: methanol (95:5) and analyzed on a one-dimensional GC equipped with a nitrogen phosphorus detector. The lowest detectable concentration was approximately 0.02 ng/mL. It would be interesting to differentiate between the two stereo-isomers of VX. In the final report of the previous grant DAMD 17-97-2-7001 a method was described using the off-line separation of VX enantiomers on a straight-phase chiral HPLC column and detection with GC-NPD. The lowest detectable concentration was approximately 1 ng/mL in that study. In this study it was decided to measure only the concentration of total (±)-VX because:

- it was anticipated that the expected concentration of VX would drop rapidly below the lowest detectable concentration,
- the stereoselectivity of the elimination of VX enantiomers seems insignificant in comparison with that of the G-agents,
- both VX enantiomers are rather toxic in view their high inhibition rate constants towards AChE.

III.4.2 Toxicokinetics of (±)-VX in HuBuChE-pretreated hairless guinea pigs

The toxicokinetics of VX were only measured in HuBuChE- pretreated hairless guinea pigs, since the toxicokinetics in non-pretreated animals were measured recently within the framework of grant DAMD 17-97-2-7001. Hairless guinea pigs were i.m. injected with HuBuChE 24 h before the injection of VX. The dose of HuBuChE was the same as described in the other intravenous toxicokinetic experiments, which means that the dose of HuBuChE is 0.7 times the dose corresponding with 2 LD50 of soman. As calculated in section III.2 and II.3 the HuBuChE dose should be 210 nmol/kg. The dose of HuBuChE of animals 1, 2 and 3 was 200 nmol/kg, whereas the dose in animals 4, 5 and 6 was only 130 nmol/kg. At 24 h after i.m. injection of the enzyme, the animals were anesthetized, artificially ventilated and atropinized. VX (56 µg/kg) was injected in the vena jugularis. Blood samples were taken at time point 0, 1, 2, 4, 10, 20, 40, 60, 120, 180 and 240 min. The size of the blood samples was 200 µl for the time points 0-60 min and 500 µl for the other blood samples. The final blood sample was more than 1 mL and was used for the fluoride reactivation assay. The results of the measurements are shown in Table 12. VX could be measured up to 240 min after administration. The concentration-time curve of (±)-VX could be fitted to a bi-exponential equation (see Figure 10) and the toxicokinetic parameters are shown in Table 13. Because the dosage of HuBuChE differed between the two groups it was decided to plot these groups in separate curves and calculate the toxicokinetic parameters also for these two different groups. Figure 11 shows VX concentration-time curves. The concentration BuChE in blood decreases very rapidly after the injection of VX.

Table 12 Concentration of (±)-VX (ng/mL) and BuChE (nM) in blood of HuBuChE pretreated, anesthetized, atropinized and artificial ventilated hairless guinea pigs after i.v. administration of VX (56 µg/kg) corresponding with 2 LD50.

Animal #	GP 1, 320 g		GP 2, 395 g		GP 3, 382 g		GP 4, 434 g		GP 5, 430 g		GP 6, 425 g		mean	SEM
HuBuChE (nmol/kg)	200		200		200		130		130		130			
Time (min)	VX (ng/mL)	BuChE (nM)	VX (ng/mL)	BuChE (nM)	VX (ng/mL)	BuChE (nM)	VX (ng/mL)	BuChE (nM)	VX (ng/mL)	BuChE (nM)	VX (ng/mL)	BuChE (nM)	VX (ng/mL)	
0	<LOD	619	<LOD	458	<LOD	764	<LOD	321	<LOD	420	<LOD	477	<LOD	
1	32.7	132	34.5	148	37.1	150	64.6	63	36.7	58	72.1	57	46.2	7.07
2	21.9	102	13.6	148	23.2	102	23.5	38	28.1	41	33.7	54	24.0	2.74
4	16.4	75	9.40	128	13.5	75	14.8	28	22.1	37	18.5	35	15.8	1.78
10	11.5	35	6.83	81	10.0	37	9.85	12	15.6	11	10.5	11	10.7	1.17
20	6.61	18	5.68	51	7.50	22	7.52	n.m.	10.6	n.m.	6.77	8	7.46	0.70
40	3.81	14	3.36	40	4.43	8	4.58	n.m.	6.32	n.m.	3.50	7	4.33	0.44
60	2.15	13	2.21	28	2.56	8	2.72	n.m.	3.16	n.m.	2.36	11	2.53	0.15
120	0.46	25	1.30	27	0.75	9	0.58	n.m.	0.49	n.m.	0.95	10	0.76	0.13
180	0.09	30	0.64	23	0.14	9	0.30	n.m.	0.12	n.m.	0.27	10	0.26	0.08
240	0.02	39	x	x	x	x	0.08	n.m.	<LOD	n.m.	0.08	27	0.06	0.02
F-reac	106						61		82		92		85	9.0

x= animal died in experiment, F-reac = reactivated ethyl sarin (ng/mL plasma) found after fluoride reactivation in sample drawn after 240 min. LOD = limit of detection (20 pg/mL), n.m. = not measured

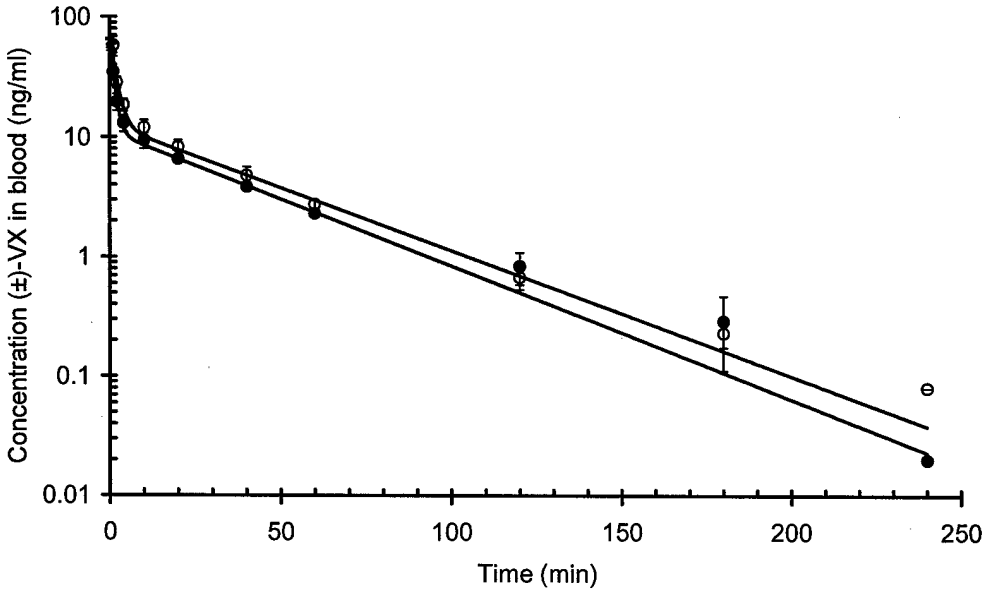

Figure 10 Mean concentration-time courses of (±)-VX (ng/mL ± SEM) in blood of HuBuChE-pretreated (○, 200 nmol/kg; ●, 130 nmol/kg) anesthetized, atropinized and artificially ventilated hairless guinea pigs after i.v. administration of (±)-VX (56 µg/kg) corresponding with 2LD50.

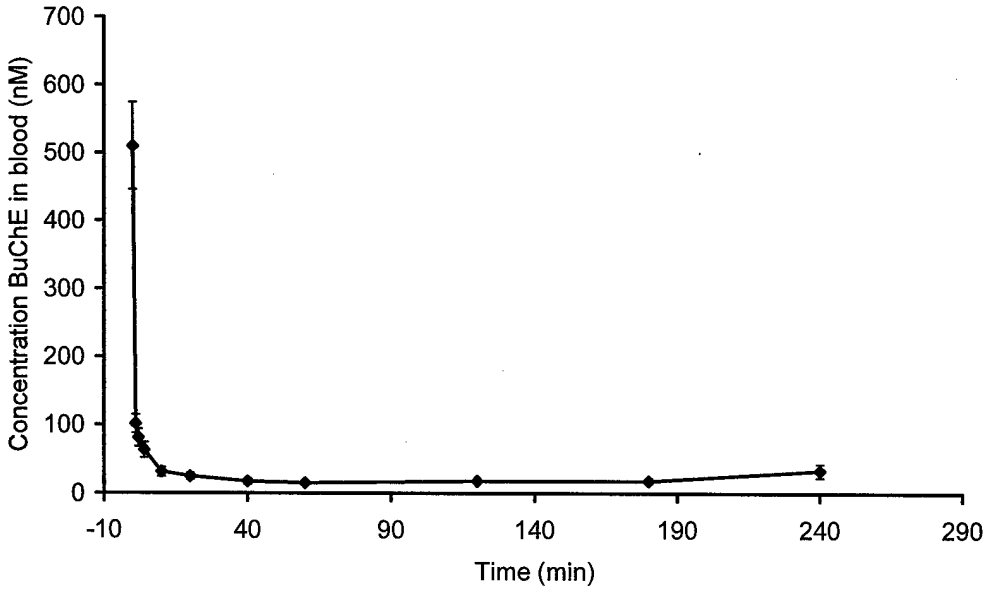

Figure 11 Mean concentration time courses of BuChE (nM ± SEM) in blood of HuBuChE pretreated (130-200 nmol/kg) anesthetized, atropinized and artificial ventilated hairless guinea pigs after i.v. administration of (±)-VX (56 µg/kg) corresponding with 2 LD50.

Table 13 Toxicokinetic parameters for (±)-VX in anesthetized, atropinized and mechanically ventilated guinea pigs that were pretreated with HuBuChE, after i.v. bolus administration of (±)-VX at a dose corresponding with 2 LD50 (56 µg/kg).

Parameter	Dimension	HuBuChE-pretreated 200 nmol/kg	HuBuChE-pretreated 130 nmol/kg
Dose	$\mu g.kg^{-1}$	56	56
Number of exponents		2	2
A	$ng.mL^{-1}$	54.2	58.5
B	$ng.mL^{-1}$	11.01	12.7
a	min^{-1}	0.817	0.571
b	min^{-1}	0.026	0.024
AUC	$ng.min.mL^{-1}$	494	626
C0	$ng.mL^{-1}$	65.2	71.1
t½,a	min	0.848	1.21
t½,b	min	26.9	28.6
Vl	$l.kg^{-1}$	0.858	0.787
Cl	$l.min.kg^{-1}$	0.113	0.089

III.5 TOXICOKINETICS OF C(+)P(-)- and C(-)P(-)-SOMAN STEREOISOMERS IN ANESTHETIZED, AND ATROPINIZED GUINEA PIGS PRETREATED WITH HuBuChE AFTER 2-MIN NOSE-ONLY EXPOSURE TO C(±)P(±)-SOMAN VAPOR IN AIR (T.O. 6)

Within the framework of DAMD 17-90-Z-0034 the LCt50 of soman was determined to be 480 $mg.min.m^{-3}$ for an 8-min exposure. Assuming the concept of linear toxic load as a function of exposure time and vapor concentration, the concentration should be 240 $mg.m^{-3}$ for a 2-min exposure yielding the same toxic load. According to the protocol the toxicokinetics of soman should be measured in pretreated guinea pigs that are exposed to 2 LCt50 in a 2-min exposure. For a 2-min exposure the concentration of the soman vapor should be 480 $mg.m^{-3}$. It was decided to attempt to measure also the toxicokinetics of soman in non-pretreated animals. Although the animals were atropinized, they were of course not artificially ventilated which means that it would be questionable whether the animals will survive such experiments. It was decided to decrease the concentration of soman vapor to 200 $mg.m^{-3}$, in order to increase the possibility that the animals would survive the exposure.

The animals were atropinized and anesthetized and constrained in a modified Battelle tube. Blood samples were drawn just before the exposure. During the exposure blood samples were not drawn. After the exposure the animal was removed from the Battelle tube and blood samples were drawn at 2, 4, 6, 10, 20, 40 and 60 min after the start of the exposure. Blood sample volumes were 200 µl for the time points 0, 2, 4 and 6 min and 500 µl for the other time points. The volume of the final blood sample taken at 120 min after exposure was more than 1 mL. This blood sample was used for the fluoride reactivation procedure. The results are shown in Table 14.

Only two non-pretreated animals were exposed to this vapor. Both animals survived the exposure. The deviations between the two curves are very large, which means that they cannot be averaged for plotting in one toxicokinetic curve. Both curves are shown in Figure 12. AUCs were calculated for each curve and are shown in Table 15. The soman levels found in blood of animal 1 are extremely high, while the soman levels in animal 2 are also rather high compared to the data derived from the intravenous toxicokinetic experiments.

Table 14 Concentrations of C(+)P(-)- and C(-)P(-)-soman stereoisomers (ng/mL) in blood of anesthetized, atropinized guinea pigs after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air.

Animal #	GP 1, 698 g		GP 2, 540 g	
Time (min)	C(+)P(-)-soman	C(-)P(-)-soman	C(+)P(-)-soman	C(-)P(-)-soman
0				
2	22.1	25.5	0.933	3.15
4	11.1	13.7	0.488	1.24
6	3.56	4.52	0.118	0.33
10	2.25	2.60	0.081	0.073
20	1.02	0.33	0.029	0.008
40	0.949	0.17	0.039	
60	< LOD	< LOD	0.021	
120	< LOD	< LOD	0.018	
F-react ($ng.mL^{-1}$)		73.4		50.1

LOD = Limit of detection (50 pg/mL), F-react is expressed as ng regenerated soman per mL plasma

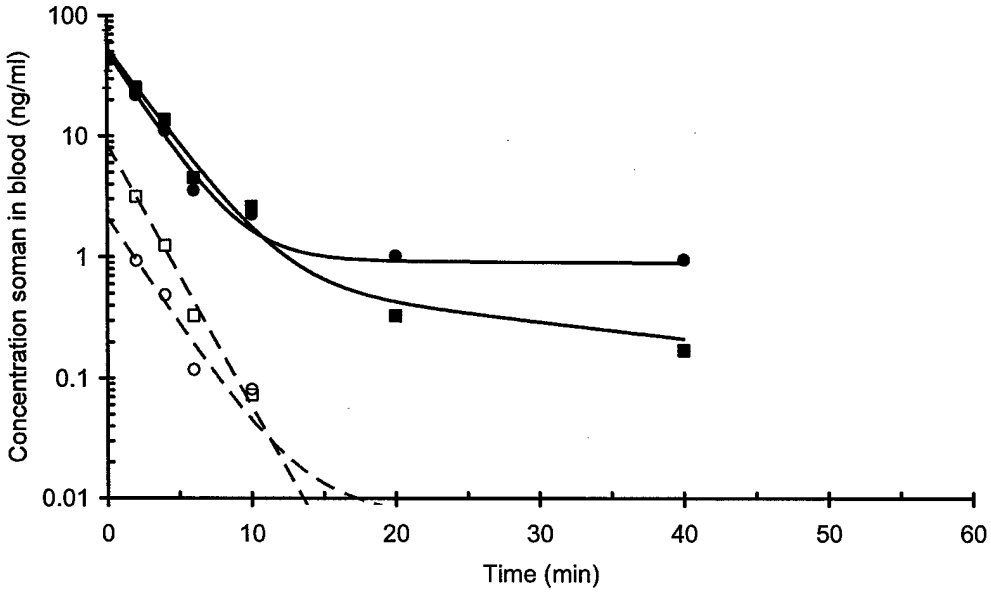

Figure 12 Concentrations of C(±)P(-)-soman stereoisomers (ng/mL) in blood of anesthetized, atropinized guinea pigs after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air. C(+)P(-)-soman GP1 (•) C(-)P(-)-soman GP1(■); C(+)P(-)-soman GP2(○), C(-)P(-)-soman GP2 (□)

Table 15 Toxicokinetic parameters of C(+)P(-)-and C(-)P(-)-soman in blood of anesthetized, atropinized guinea pigs after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air.

Animal #		1		2	
Parameter	Dimension	C(+)P(-)-soman	C(-)P(-)-soman	C(+)P(-)-soman	C(-)P(-)-soman
Number of exponents		2	2	2	1
A	$ng.mL^{-1}$	49.6	53.13	2.106	8.56
B	$ng.mL^{-1}$	0.944	0.759	0.013	
a	min^{-1}	0.421	0.376	0.408	0.498
b	min^{-1}	0.0013	0.0319	0.027	
AUC	$ng.min.mL^{-1}$	843	165	5.64	17.2

In the next series of experiments animals were pretreated with HuBuChE at 24 h before the exposure to soman vapor. According to the protocol the amount of HuBuChE that would be i.m. injected should be 0.25 times the estimated inhaled dose of soman. Assume an animal of 500 g with a respiratory minute volume of 100 mL. The respirated volume in 2 min is 200 mL which means that 200 mL * 400 ng/mL = 80,000 ng of soman is inhaled, which corresponds with 439 nmol. Assuming that only 50 % of soman vapor is retained in the lungs the real dose is 219 nmol soman. According to the protocol the dose of HuBuChE should be 25% of the inhaled dose which corresponds with 55 nmol or 110 nmol/kg. At 24 h after the i.m. administration of the

enzyme, the animals were atropinized, anesthetized and a carotid artery cannula was installed. The initial concentrations of BuChE in blood at 24 h after injection of the enzyme were in the range of the expected values. Animals were exposed for two min as described above and blood was sampled according to the schedule mentioned for the non-pretreated animals. The blood levels of soman were mostly under the detection limit. Table 16 shows the results.
It is clear that the concentrations of soman in blood are much lower than in the non-pretreated animals. It is also clear that the deviation between the animals is too high to construct one averaged toxicokinetic curve. The separate curves of the C(-)P(-)-soman concentrations are shown in Figure 13. The majority of the data could not be fitted to a normal exponential curve, characteristic for an elimination process. The AUCs were calculated using the trapezoidal method. These data were also not averaged because there were some doubts about the reproducibility of the exposures. It was observed that some animals had difficulties with breathing during the exposure (animals # 4, 8 and 9). Unfortunately, the respiration was not measured during the exposure. It is expected that when an animal breathes normally during the exposure, it will inhale a large amount of soman which results in a high AUC and low residual BuChE activity in blood. If an animal breathes shallowly a small amount of soman will be inhaled which results in a low AUC and high residual BuChE activity in blood. The amount of soman that could be regenerated with fluoride is also a measure for the inhaled dose. From that data it is verified that animals 4, 8 and 9 inhaled a lower dose of soman.

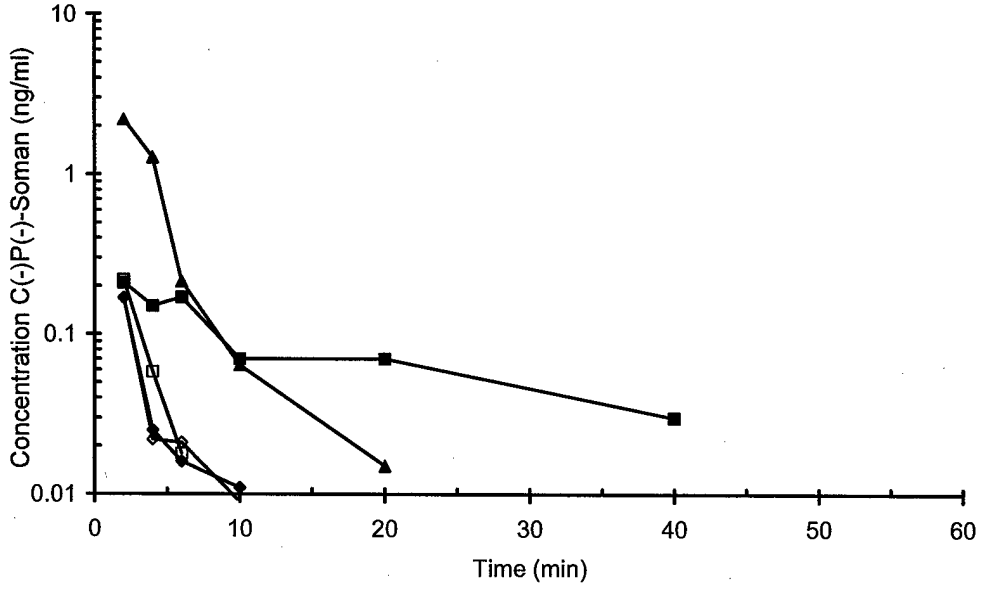

Figure 13 Concentration of C(-)P(-)-soman (ng/mL) in blood of anesthetized, atropinized and HuBuChE-pretreated guinea pigs (100 nmol/kg) after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air. GP3(♦), GP4 (■), GP5 (▲), GP6 (□), GP7 (◊)

Table 16 Concentrations of C(+)P(-)- and C(-)P(-)-soman (ng/mL) and BuChE (nM) in blood of anesthetized, atropinized and HuBuChE-pretreated guinea pigs (100 nmol/kg) after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air.

Animal	GP 3, 528 g			GP 4, 585 g			GP 5, 605 g			GP 6, 540g		
Time (min)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)
0	< LOD	< LOD	435	< LOD	< LOD	395	< LOD	< LOD	516	< LOD	< LOD	503
2	0.016	0.168	115	< LOD	0.21	201	0.054	2.19	40	< LOD	0.218	264
4	0.029	0.025	111	< LOD	0.15	178	0.156	1.27	13	< LOD	0.058	177
6	< LOD	0.016	95	< LOD	0.17	145	0.07	0.214	13	< LOD	0.018	181
10	< LOD	0.011	120	< LOD	0.07	121	0.057	0.064	-1	< LOD	< LOD	154
20	< LOD	< LOD	101	< LOD	0.07	86	0.045	0.015	13	< LOD	< LOD	186
40	< LOD	< LOD	91	< LOD	0.03	71	< LOD	< LOD	18	< LOD	< LOD	167
60	< LOD	< LOD	86	< LOD	< LOD	58	< LOD	< LOD	22	< LOD	< LOD	179
AUC		0.28			2.86			6.05			0.388	
F-react		73.9			20.2			81			63.3	

LOD = limit of detection (50 pg/mL), AUC is expressed in $ng.min.mL^{-1}$, F-react is expressed as ng regenerated soman per mL plasma

Table 16 (Continued) Concentrations of C(+)P(-)- and C(-)P(-)-soman (ng/mL) and BuChE (nM) in blood of anesthetized, atropinized and HuBuChE-pretreated guinea pigs (100 nmol/kg) after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air.

Animal	GP 7, 630 g			GP 8, 640 g			GP 9, 650 g		
Time (min)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)
0	< LOD	< LOD	565	< LOD	< LOD	461	< LOD	< LOD	370
2	< LOD	0.169	181	< LOD	0.032	332	< LOD	< LOD	301
4	< LOD	0.022	173	< LOD	< LOD	251	< LOD	< LOD	231
6	< LOD	0.021	162	< LOD	< LOD	279	< LOD	< LOD	235
10	< LOD	0.009	172	< LOD	< LOD	279	< LOD	< LOD	223
20	< LOD	< LOD	166	< LOD	< LOD	194	< LOD	< LOD	219
40	< LOD	< LOD	159	< LOD	< LOD	255	< LOD	< LOD	209
60	< LOD	< LOD	159	< LOD	< LOD	219	< LOD	< LOD	185
AUC		0.339			-			-	
F-react		53.3			32.7			30.6	

F-react is expressed as ng regenerated soman per mL plasma, AUC is expressed in $ng.min.mL^{-1}$, LOD = limit of detection (50 pg/mL)

III.6 TOXICOKINETICS OF C(+)P(-)- AND C(-)P(-)-SOMAN STEREOISOMERS IN ANESTHETIZED AND ATROPINIZED GUINEA PIGS PRETREATED WITH HuBuChE DURING AND AFTER 300 MIN NOSE-ONLY EXPOSURE TO C(±)P(±)-SOMAN VAPOR IN AIR (T.O. 7)

In another series of experiments, guinea pigs pretreated with HuBuChE were exposed to low level concentrations of soman vapor. According to the protocol HuBuChE-pretreated animals would be exposed to 2 LCt50 during an exposure time of 300 min. The LCt50 of soman for guinea pigs was determined within the framework of DAMD 17-90-Z-0034 and was 480 $mg.min.m^{-3}$. For a 300-min exposure the desired concentration would be 3.2 $mg.m^{-3}$ to reach a dose of 2 LCt50. It was decided to generate soman vapor with a concentration of 2 $mg.m^{-3}$ in order to prevent that animals would die during the exposure.
Guinea pigs were pretreated with HuBuChE (100 nmol/kg). After 24 h the animals were anesthetized, atropinized and a carotid cannula was installed. The levels of BuChE after 24 h were as expected, which means that about 25% of the administered dose of HuBuChE circulated in blood at the time of exposure. During the exposure blood samples were drawn through the cannula and processed and analyzed with 2D-GC. The total volume of each blood sample was 500 µl. From each blood sample 10 µl was used for the Ellman assay to determine BuChE activity. A portion of 300 µl blood was used to determine intact soman. The other part (200 µl) was used in the fluoride reactivation assay. By means of fluoride reactivation it could be determined whether the animal inhaled any soman.
The results are shown in Table 17. Intact soman was almost not detected, indicating that the inhaled soman was eliminated by the scavenger. The amount of regenerable soman was rather high. The inhibition of BuChE was also an indicator for soman inhalation. Table 17 shows that intact soman was only detectable when BuChE was almost completely inhibited. Toxicokinetic curves of intact soman could not be constructed and AUC's could not be calculated. The amount of regenerable soman was plotted vs. time in Figure 14. It must be mentioned that all animals died during the exposure. It is not likely that animal 1 died because of soman intoxication in view of the high levels of residual BuChE, while it is likely that the other animals died because of soman intoxication in view of their low amount of residual BuChE.

Table 17 Concentrations of C(+)P(-)- and C(-)P(-)-soman (ng/mL blood), fluoride regenerated soman (ng/mL plasma) and BuChE (nM) in blood of anesthetized, atropinized and HuBuChE-pretreated guinea pigs (100 nmol/kg) during and after a 300-min nose-only exposure to 2 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air.

Animal	GP 1, 515 g				GP 2, 550 g			
Time (min)	C(+)P(-)-soman	C(-)P(-)-soman	F-react	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	F-react	BuChE (nM)
0	<LOD	<LOD	< LOD	432	<LOD	<LOD	< LOD	399
30	<LOD	<LOD	15	387	<LOD	<LOD	26	352
60	<LOD	<LOD	16	312	<LOD	<LOD	44	246
120	<LOD	<LOD	31	252	<LOD	<LOD	52	126
180	<LOD	<LOD	35	230	<LOD	<LOD	120	1
240	dead				0.293	0.421	132	1
300								1

LOD = limit of detection (50 pg/mL), F-react is expressed as ng regenerated soman per mL plasma

Table 17 (Continued) Concentrations of C(+)P(-)- and C(-)P(-)-soman (ng/mL blood), fluoride regenerated soman (ng/mL plasma) and BuChE (nM) in blood of anesthetized, atropinized and HuBuChE-pretreated guinea pigs (100 nmol/kg) during and after a 300-min nose-only exposure to 2 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air.

Animal	GP 3, 595 g				GP 4, 627 g			
Time (min)	C(+)P(-)-soman	C(-)P(-)-soman	F-react	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	F-react	BuChE (nM)
0	<LOD	<LOD	<LOD	425	<LOD	<LOD	<LOD	517
30	<LOD	<LOD	22	288	<LOD	<LOD	18	337
60	<LOD	<LOD	52	89	<LOD	<LOD	40	152
120	<LOD	<LOD	84	2	0.24	0.42	113	2
180				1	< LOD	0.39	67	2
240	dead				dead			
300								

LOD = limit of detection (50 pg/mL), F-react is expressed as ng regenerated soman per mL plasma

Although it was not proposed in the protocol, one animal that was not pretreated with HuBuChE was also exposed to the same conditions as described above. Intact soman was detected after 120 min. The level of BuChE decreased from 16 nM to a critical 3 nM and the animal was dead when the sample for time point 180 min was to be taken. Unfortunately the analysis for the determination of the fluoride reactivated soman for sample t=120 failed. It can be concluded that the animals that were pretreated with HuBuChE lived at least 1 h longer.

Table 18 Concentrations of C(+)P(-)- and C(-)P(-)-soman (ng/mL blood), fluoride regenerated soman (ng/mL plasma) and BuChE (nM) in blood of anesthetized, atropinized guinea pigs during and after a 300-min nose-only exposure to 2 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air.

Animal #	GP 5, 650 g			
Time	C(+)P(-)-soman	C(-)P(-)-soman	F-react	BuChE (nM)
0	< LOD	< LOD	<LOD	16
30	< LOD	< LOD	24	10
60	< LOD	< LOD	35.7	3
120	< LOD	0.082	failed	3
180			dead	

LOD = limit of detection (50 pg/mL), F-react is expressed as ng regenerated soman per mL plasma

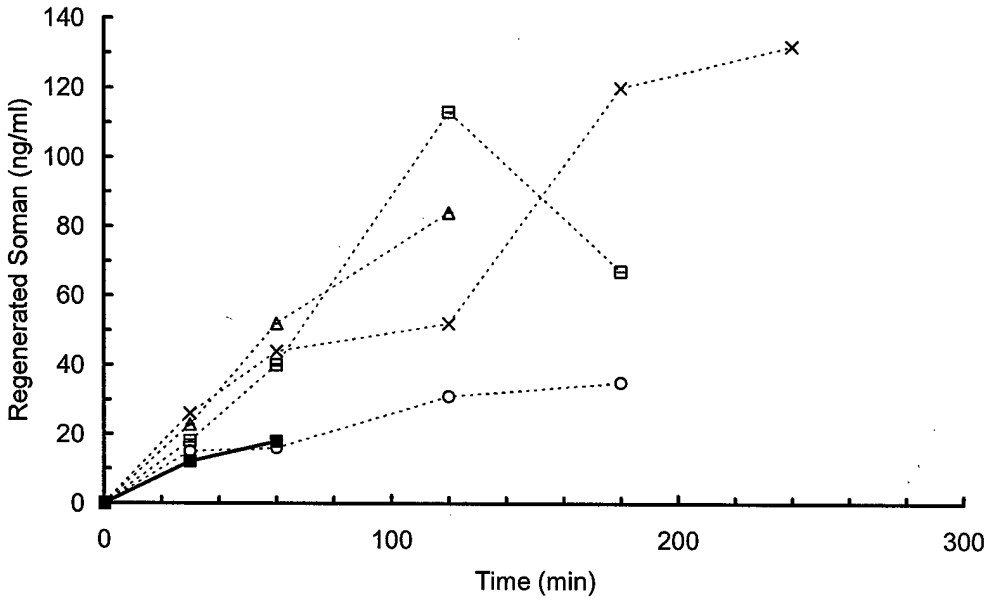

Figure 14 Concentrations of fluoride regenerated soman in plasma (ng/mL) of anesthetized, atropinized and HuBuChE-pretreated guinea pigs (100 nmol/kg) during a 300-min nose-only exposure to 2 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air. Solid line (■) non pretreated animal.

III.7 TOXICOKINETICS OF (±)-SARIN STEREOISOMERS IN ANESTHETIZED, AND ATROPINIZED GUINEA PIGS PRETREATED WITH HuBuChE AFTER 2-MIN NOSE-ONLY EXPOSURE TO (±)-SARIN VAPOR IN AIR (T.O. 8)

Within the context of cooperative agreement DAMD17-90-Z-0034 we have determined the 24-h LC50 of (±)-sarin in anesthetized and atropinized guinea pigs for an 8-min nose-only exposure (Benschop and Van Helden, 1993). The LC50 was established to be 47 $mg.m^{-3}$ (95-% confidence intervals 44-50 $mg.m^{-3}$). For a 2-min exposure this corresponds with an LCt50-value of 376 $mg.min.m^{-3}$. For the purpose of the current study we proposed to expose guinea pigs to 2 LCt50 in 2 min. Assuming that Haber's rule would apply, i.e., that any combination of concentration and exposure time would yield an identical biological response at the same C*t-value, this would imply nose-only exposure of the guinea pigs to a concentration of (±)-sarin vapor in air of 376 $mg.m^{-3}$ for 2 min. Due to the high volatility of (±)-sarin, generating such a concentration was no problem at all. The guinea pigs were pretreated with the same dose of HuBuChe as calculated for the toxicokinetic experiments with soman (see section III.5).
Upon exposure of three HuBuChE-pretreated guinea pigs to the high concentration of sarin (376 $mg.m^{-3}$) in order to study the toxicokinetics, two out of three animals died before the planned end point of the experiment. The results are presented in Table 19 and Figure 15.

Guinea pigs GP 9, 10 and 11 received doses of HuBuChE that were rather close to the desired 54 nmol (see Table 19). However, since the body weight of these animals was in the range of 658-685 g instead of 500 g, they should have been pretreated with 71-74 nmol of HuBuChE. Actually, these animals were pretreated with a dose corresponding with ca. 5000 U/kg. For practical purposes it was decided to continue pretreatment with a HuBuChE dose of *ca.* 5000 U/kg throughout the inhalation experiments.

Table 19 Concentrations of (+)- and (-)-sarin stereoisomers (ng/mL) and BuChE (nM) in blood of anesthetized, atropinized and HuBuChE-pretreated guinea pigs after a 2-min nose-only exposure to 376 $mg.m^{-3}$ of (±)-sarin vapor in air.

Animal #	GP 9, 658 g		GP 10, 683 g a			GP 11, 685 g b		
HuBuChE dose (nmol)	55.6		56.9			56.7		
Time (min)	(-)-sarin	BuChE	(+)-sarin	(-)-sarin	BuChE	(+)-sarin	(-)-sarin	BuChE
0	< LOD	298	< LOD	< LOD	386	< LOD	< LOD	361
2	0.033	286	< LOD	27.3	96	0.97	35.3	37
4	0.087	208	2.7	25.6	80	2.16	25.3	35
10	0.123	97	0.44	5.83	78	0.023	1.12	35
20	0.060	88	0.024	0.118	10	0.016	0.131	12
40	0.114	97	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
60	0.11	105	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AUC	5.6	6788		178			147	
F-react	270			375			528	

LOD = limit of detection (40 pg/mL); n.s. = not sampled, AUC is expressed in $ng.min.mL^{-1}$

[a] Animal died shortly after taken 20-min sample

[b] Animal died around 30 min

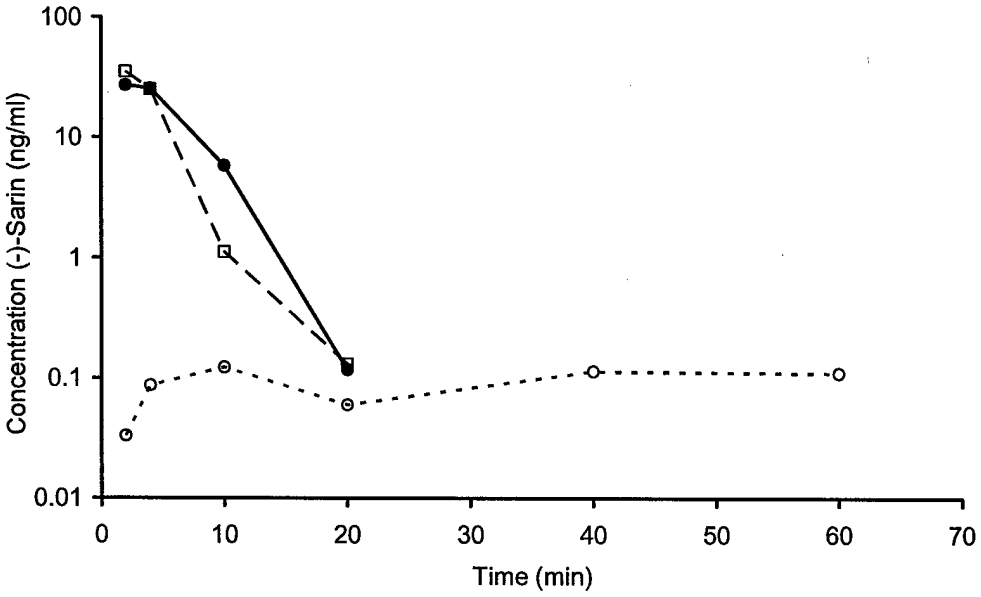

Figure 15 Concentration-time courses of (-)-sarin (ng/mL) in blood of anesthetized, atropinized, and HuBuChE-pretreated guinea pigs after a 2-min nose-only exposure to 376 $mg.m^{-3}$ of (±)-sarin vapor in air. ○ = guinea pig GP 9, ● = guinea pig GP 10, □ = guinea pig GP 11.

The only animal that survived the 60-min experiment, GP 9, was breathing very shallow and irregularly in comparison with the other two animals. This implies that GP 9 inhaled less of the (±)-sarin vapor during the 2-min exposure than GP 10 and GP 11, which provides for a tentative explanation why this animal survived the experiment. The observed lower concentrations of (-)-sarin and higher residual HuBuChE concentration in blood of GP 9 are in agreement with this hypothesis. It is interesting to note that in animals GP 10 and 11 (+)-sarin could be detected in blood after the nose-only exposure to (±)-sarin. In previously performed inhalation studies involving nose-only exposure to 0.4 and 0.8 LCt50 (±)-sarin in 8 min, the (+)-sarin isomer was not detectable in the blood samples (Benschop and Van Helden, 1993).

The fact that two out three animals died within 30 min after exposure to 376 $mg.m^{-3}$ of (±)-sarin for 2 min, suggests that Haber's rule is not applicable under these conditions. Since we felt it would not be feasible to perform a toxicokinetic study of a reasonable time span (60 min) in this way, unless we would be willing to use considerably more animals than planned, we decided to lower the (±)-sarin vapor concentration to 200 $mg.m^{-3}$.
Both naive and HuBuChE-pretreated animals were nose-only exposed to this concentration for 2 min, and blood samples were drawn up to 60 min after the start of the exposure for analysis of the concentrations of (±)-sarin stereoisomers and BuChE activity. The results are presented in Tables 20 and 21, and in Figures 16 and 17.

Table 20 Concentrations of (±)-sarin stereoisomers (ng/mL) in blood of anesthetized and atropinized guinea pigs after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of (±)-sarin vapor in air.

Animal #	GP 12, 713 g		GP 15, 617 g		GP 16, 633 g		GP 18, 768 g		GP 21, 668 g	
Time (min)	(+)-sarin	(-)-sarin	(+)-sarin	(-)-sarin	(+)-sarin	(-)-sarin	(+)-sarin	(-)-sarin	(+)-sarin	(-)-sarin
0	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2	0.025	0.22	< LOD	0.48	< LOD	0.11	0.56	16.4	2.26	19.7
4	0.018	0.51	0.058	4.64	< LOD	0.48	0.58	6.14	0.091	6.14
10	< LOD	0.14	< LOD	0.81	< LOD	0.28	< LOD	0.62	0.078	1.40
20	< LOD	0.093	< LOD	0.31	< LOD	0.092	< LOD	0.12	< LOD	0.20
40	< LOD	0.080	< LOD	0.077	< LOD	0.11	< LOD	0.09	< LOD	0.076
60	< LOD	0.079	< LOD	0.064	< LOD		< LOD	0.12	< LOD	0.097
AUC		7.1		32.5		7.95		50.8		60.9
F-react		72		50		69		38		53

LOD = limit of detection (40 pg/mL); n.s. = not sampled, AUC is expressed in $ng.min.mL^{-1}$, F-react is expressed as ng regenerated sarin per mL plasma

Table 21 Concentrations of (+)- and (-)-sarin (ng/mL) and BuChE (nM) in blood of anesthetized, atropinized and HuBuChE-pretreated guinea pigs after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of (±)-sarin vapor in air.

Animal #	GP 13, 536 g			GP 14, 540 g			GP 17, 674 g		GP 19, 768 g			GP 20, 648 g		
HuBuChE dose (nmol)	42.6			45			49.4		65			65		
Time (min)	(+)-sarin	(-)-sarin	BuChE	(+)-sarin	(-)-sarin	BuChE	(-)-sarin	BuChE	(+)-sarin	(-)-sarin	BuChE	(+)-sarin	(-)-sarin	BuChE
0	< LOD	< LOD	296	< LOD	< LOD	302	< LOD	321	< LOD	< LOD	412	< LOD	< LOD	437
2	< LOD	< LOD	140	0.29	1.38	171	0.075	171	0.071	0.53	191	0.67	1.99	101
4	< LOD	< LOD	260	< LOD	0.33	109	0.23	120	0.17	0.62	61	0.016	3.06	17
10	< LOD	0.047	102	< LOD	0.046	25	0.17	102	0.14	0.31	105	< LOD	0.62	28
20	< LOD	0.079	137	< LOD		41	0.13	58	< LOD	0.13	79	< LOD	0.080	26
40	0.06	0.14	93	< LOD		59	0.27	40	< LOD	0.12	66	< LOD	0.059	26
60		0.107	74	< LOD		31	0.14	60	< LOD		73	< LOD	0.058	26
AUC					3.97		11.1			9.84			22.1	
F-react		266					211			264			225	

< LOD = not detectable; P(+)-sarin was not detected in GP 17. AUC is expressed in $ng.min.mL^{-1}$, F-react is expressed as ng regenerated soman per mL plasma

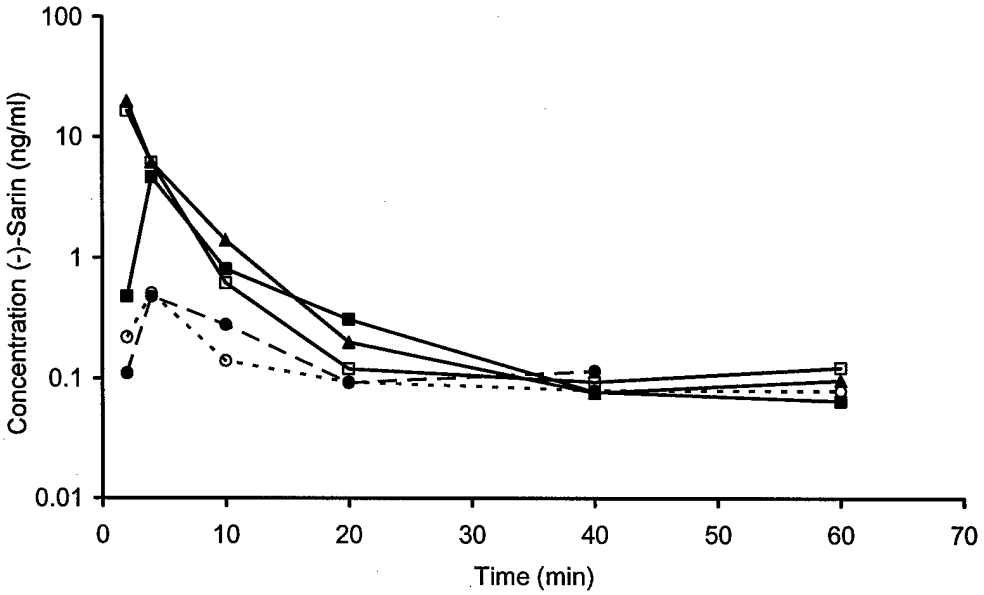

Figure 16 Concentration-time courses of (-)-sarin (ng/mL) in blood of anesthetized and atropinized guinea pigs after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of (±)-sarin vapor in air. ○ = guinea pig GP 12, ■ - guinea pig GP 15, ● = guinea pig GP 16, □ = guinea pig GP 18, ▲ = guinea pig GP 21.

From the data presented in Table 20 and Figure 16 it is clear that the concentration of (-)-sarin increases during the 2-min exposure, and in some animals (GP 12, GP 15, GP 16) continues to increase in the 2 min after ending the exposure. Such an increase in blood concentration of (-)-sarin after termination of the exposure was not observed for 8-min exposure to 0.4 and 0.8 LCt50 (±)-sarin (Benschop and Van Helden, 1993) and neither for a 2-min exposure to soman (Cf. Table 14). Furthermore, it is remarkable that in most of the animals the (+)-sarin isomer is measurable in the first few minutes after ending the 2-min exposure, which - as already mentioned above - was not the case in the 8-min exposure studies.

The data presented in Table 20 and Figure 16 also show that there is more than a tenfold difference between the highest and the lowest curves measured in these five animals, at least in the time period up to 10-20 min. Subsequently, all (-)-sarin concentrations are in the same range. Since the generated (±)-sarin vapor concentrations were all within the range of 200 ± 20 $mg.m^{-3}$, the variation between the curves is likely the result of differences in respiratory minute volumes and respiratory frequencies between the animals. Unfortunately, these parameters have not been measured.

Due to the large variation, it is probably not allowed to derive one mean concentration-time curve for these five experiments. The AUC has been determined by numerical integration for all experiments. The last of table 20 line gives the value of fluoride regenerated sarin in the final blood sample.

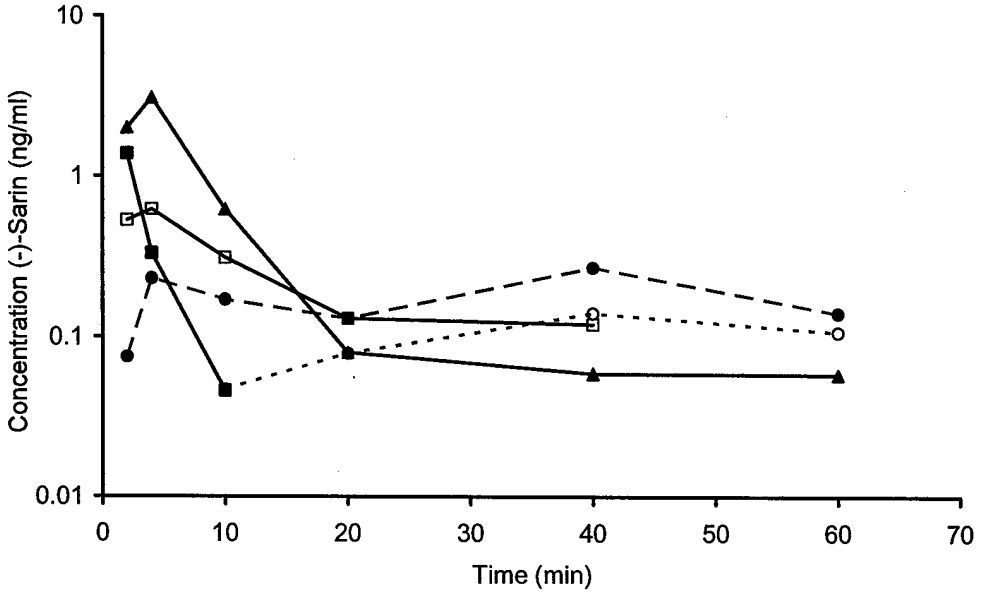

Figure 17 Concentration-time courses of (-)-sarin (ng/mL) in blood of anesthetized, atropinized and HuBuChE-pretreated guinea pigs after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of (±)-sarin vapor in air. ○ = guinea pig GP 13, ■ = guinea pig GP 14, ● = guinea pig GP 17, □ = guinea pig GP 19, ▲ = guinea pig GP 20.

The data presented in Table 21 and Figure 17 show that also in the HuBuChE-pretreated animals the concentration of (-)-sarin increases during the 2-min exposure, and in some animals (GP 17, GP 19, GP 20) continues to increase in the 2 min after ending the exposure. Such retarded uptake in the respiratory tract is not observed in the case of soman under comparable conditions (Cf. Table 14). Furthermore, in most of the animals the (+)-sarin isomer is measurable in the first few minutes after ending the 2-min exposure. The BuChE activity is reduced considerably during the 2-min exposure and thereafter. As was also the case after intravenous administration of (±)-sarin to HuBuChE-pretreated animals, at the end of the toxicokinetic experiment there is still quite some residual BuChE activity left in the blood, whilst low concentrations of (-)-sarin are still circulating.

Like in the naive animals, there is more than a tenfold difference between the highest and the lowest curves measured in these five animals, in this case for the whole duration of the toxicokinetic experiment. Since the generated (±)-sarin vapor concentrations were all within the range of 200 ± 20 $mg.m^{-3}$, the observed variation between the curves is likely to be due to differences in respiratory minute volumes and respiratory frequencies between the animals, which we did not measure.

Although the concentration-time curves of the HuBuChE-pretreated animals tend to be somewhat lower than those measured in the naive animals, the difference is not dramatic. The differences between the pretreated and non-pretreated is more apparent when the AUC of the various toxicokinetic experiments are compared with each other. The values are mentioned in the last lines of Table 20 and 21. The average AUC for the non-pretreated animals was 31 ± 11 $ng.min.mL^{-1}$, while the average AUC for the pretreated animals was 11.8 ± 4 $ng.min.mL^{-1}$. The difference between pretreated and non-pretreated animals is also visible in the amount of sarin that could be regenerated with fluoride in the final blood sample.

III.8 TOXICOKINETICS OF (±)-VX IN ANESTHETIZED, ATROPINIZED AND ARTIFICIALLY VENTILATED HAIRLESS GUINEA PIGS PRETREATED WITH HuBuChE AFTER PERCUTANEOUS APPLICATION (T.O. 9)

The toxicokinetics of VX were measured in HuBuChE pretreated hairless guinea pigs after percutaneous application. The percutaneous toxicokinetics of VX in non-pretreated animals were measured within the framework of a previous grant DAMD 17-97-2-7001. One of the conclusions from that study was that the spread of the concentrations of intact VX in blood was very high. The maximum concentration VX in blood was reached not earlier than 5 h after application and the levels were not higher than 0.2 ng/mL. In four out of nine animals, hardly any VX could be detected. It was decided to challenge the animals with a dose of VX corresponding with 2 LD50 (= 250 µg/kg). According to the protocol the dose of HuBuChE was the same as used for the intravenous toxicokinetic experiments (100 nmol/kg). At 24 h after i.m. injection of the enzyme, the animals were anesthetized, atropinized, artificially respirated and a carotid cannula was inserted. VX in isopropyl alcohol solution (<15 µl) was applied onto the belly of the hairless guinea pig. Blood samples (0.5 mL) were drawn at 0, 1, 3, 5, 6, 7 and 8 h and were processed and analyzed with GC. The final blood sample was also processed according to the fluoride reactivation procedure. The corresponding phosphofluoridate, ethyl sarin, was analyzed with GC-MS. Table 22 shows the results.

VX was not detected in any of the blood samples, which means that the level of VX was lower than 40 pg/mL. Indirect proof of VX penetration was found by the decrease of BuChE concentration in blood, whereas direct proof of VX penetration was obtained by the fluoride regeneration assay. The concentrations of regenerated ethyl sarin were > 10 ng/mL plasma. One animal was not pretreated with HuBuChE. VX could not be detected in blood of this animal, but a reduction of BuChE activity was observed. The amount of regenerated ethyl sarin was < 1 ng/mL. Ethyl sarin could only be detected using thermodesorption-GC-MSD allowing introduction of a larger sample volume onto the GC.

Table 22 Concentrations of (±)-VX (ng/mL), regenerated ethyl sarin (ng/mL) and total blood BuChE (nM) in blood of anesthetized, atropinized and artificially ventilated hairless guinea pigs that were pretreated with HuBuChE (100 nmol/kg) after a percutaneous application corresponding with 2 LD50 (250 µg/kg).

Animal	GP 9, 498 g		GP 10, 550 g		GP 11, 670 g		GP 12, 581 g		GP 13, 670 g		GP 14, 580 g	
Time (h)	VX	BuChE	VX	BuChE	VX	BuChE	VX	BuChE	VX	BuChE	VX	BuChE
0	< LOD	319	< LOD	212	< LOD	126	< LOD	340	< LOD	346	< LOD	18
1	< LOD	333	< LOD	185	< LOD	142	< LOD		< LOD	357	< LOD	18
2	< LOD	414	< LOD		< LOD		< LOD		< LOD		< LOD	
3	< LOD	321	< LOD	155	< LOD	147	< LOD	329	< LOD	336	< LOD	17
4	< LOD	311	< LOD		< LOD	125	< LOD	304	< LOD	294	< LOD	15
5	< LOD	325	< LOD	127	< LOD	91	< LOD	277	< LOD	285	< LOD	12
6	< LOD		< LOD	131	< LOD	59	< LOD	252	< LOD	268	< LOD	9
7	< LOD	259	< LOD	132	< LOD		< LOD	239	< LOD	241	< LOD	9
8	< LOD		< LOD	130	< LOD		< LOD		< LOD		< LOD	
9	< LOD		< LOD	116	< LOD		< LOD		< LOD		< LOD	
AUC												
F-react	11				19		16				<1	

LOD = limit of detection (40 pg/mL), F-react is expressed in ng regenerated ethyl sarin / mL plasma

III.9 TIME COURSE OF HuBuChE IN BLOOD OF THE MARMOSET FOLLOWING INTRAMUSCULAR ADMINISTRATION (T.O. 10)

In order to establish which point in time after administration of HuBuChE to marmosets would be optimal for a nerve agent challenge, the time course of BuChE activity was determined in blood samples drawn from three marmosets at various time points after administration of the enzyme, using the colorimetric method of Ellman *et al.* (1961). The results are shown in Table 23. After i.m. administration the BuChE concentration in blood increases rapidly with a half life of approximately 2.8 h. Subsequently the activity declines with a half life of ca 60 h. The deviation between the measurements was reasonable which means that it was possible to construct a pharmacokinetic curve with the following equation: $BuChE\ activity = A*exp(-k_{el}*t)-B*exp(-k_{abs}*t)+C$. The curve is shown in Figure 18. The maximum concentration BuChE in blood was reached after between 12 -16 h. At that time about 23 % of the injected dose HuBuChE circulated in the blood. Based on these results it was decided to inject the marmosets with the enzyme at 16 h before the challenge with nerve agent.

Table 23 Activity of BuChE (nM) in blood of marmosets at various time points after intramuscular administration of HuBuChE.

Animal Dose (nmol)	MS 1, 317 g 35	MS 2, 397 g 35	MS 3, 416 g 35	Mean ± SEM
Time (hr)	BuChE	BuChE	BuChE	BuChE
0	11	14	14	13 ± 1
1	109	96	158	121 ± 19
2	219	161	186	189 ± 17
4	239	181	214	211 ± 17
8	305	262	296	288 ± 13
12	356	268	315	313 ± 26
16	273	240	325	280 ± 25
24	313	300	330	314 ± 9
48	177	215	226	206 ± 15
72	118			118
96		108	188	148 ± 33

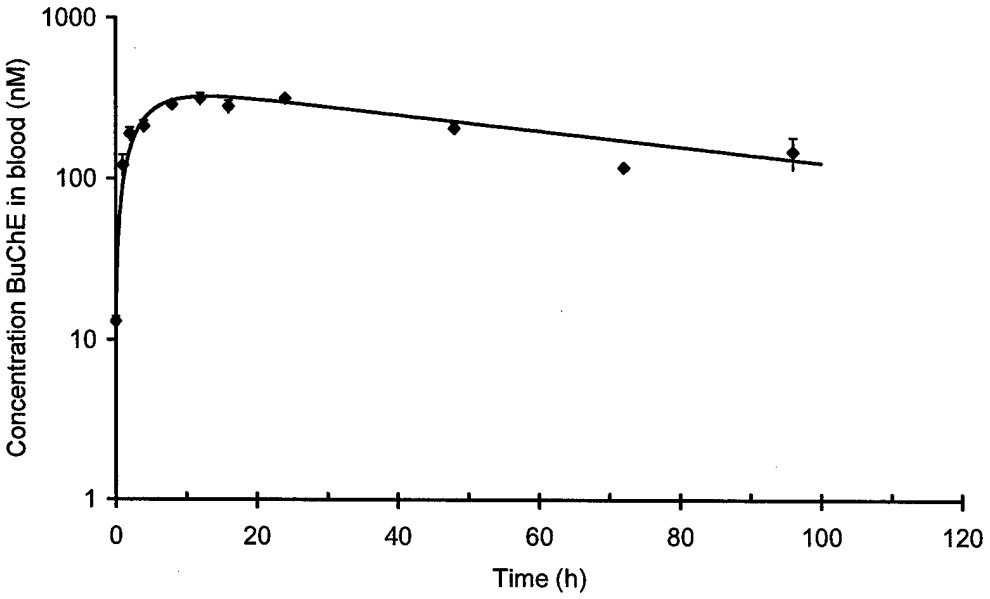

Figure 18 Concentration-time course of BuChE-activity measured in blood of marmosets (n=3), following intramuscular administration of HuBuChE (35 nmol). Data were fitted to the following equation:
[BuChE]= 390*exp(-0.013*t)-377*exp(-0.2554*t).

III.10 TOXICOKINETICS OF C(±)P(±)-SOMAN STEREOISOMERS IN ANESTHETIZED, AND ATROPINIZED MARMOSETS PRETREATED WITH HuBuChE AFTER A 5-MIN NOSE-ONLY EXPOSURE TO C(±)P(±)-SOMAN VAPOR IN AIR (T.O. 12 and 13)

It was attempted to measure the toxicokinetics of soman in marmosets after nose-only exposure. According to the protocol the non-pretreated marmosets had to be exposed for 2 min to a concentration resulting in 0.8 LCt50. The LCt50 of soman in marmosets has never been determined. The only data available is a comparison of the LD50 of guinea pigs (28 µg/kg) and marmosets (10µg/kg). Based on that ratio, the dose of soman should be 2.8 times lower than the dose used for the guinea pigs which would mean a 2 min exposure to 70 $mg.m^{-3}$. It was decided to choose the dose conservatively and to expose the marmosets 2 min to 50 $mg.m^{-3}$. Animals were anesthetized, atropinized and a carotid cannula was installed. Next, the animals were exposed nose-only for 2 min to soman vapor in air. Blood was sampled after the exposure at time points 2, 4, 10, 20, 40 and 60 min. Volumes of the blood samples were 0.2 mL for the time points 0-2 min and 0.3 mL for the remaining time points. The last blood sample was used for the fluoride reactivation assay. A total of two animals was 2-min exposed to 50 $mg.m^{-3}$ soman vapor in air. Table 24 shows the results.

Table 24 Concentrations of C(±)P(-)-soman stereoisomers (ng/mL) in blood of anesthetized and atropinized marmosets after a 2-min nose-only exposure to 50 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air.

Animal #	MS 1, 360 g		MS 2, 380 g	
Time (min)	C(+)P(-)-soman	C(-)P(-)-soman	C(+)P(-)-soman	C(-)P(-)-soman
0	< LOD	< LOD	< LOD	< LOD
2	3.41	2.91	< LOD	< LOD
4	2.25	1.93	< LOD	< LOD
10	0.45	0.49	< LOD	< LOD
20	0.133	0.13	< LOD	< LOD
40			< LOD	< LOD
60			< LOD	< LOD
AUC ($ng.min.mL^{-1}$)	14.9	13.6		
F-react ($ng.mL^{-1}$)		3.93		3.02

LOD = limit of detection (50 pg/mL), F-react is expressed in ng soman/ mL plasma

Intact soman could be measured in the blood of marmoset 1. Both P(-) isomers of soman could be measured. Remarkably, the concentration of C(+)P(-)-soman was higher than that of C(-)P(-)-soman. Soman could also be regenerated from regenerable binding sites in the blood sample that was taken 60 min after exposure. Intact soman could not be measured in blood of animal 2 which was exposed to the same soman vapor concentration. However, regenerated soman could be measured in the last blood sample indicating that the animal inhaled soman. Apparently, the amount of soman that can be regenerated is not a reliable measure for the amount of inhaled soman at high doses. Presumably, these binding sites are very rapidly saturated after inhalation of only a fraction of the offered concentration. It can be imagined that the 2-min exposure is too short to attain a reproducible exposure. Therefore, it was decided to increase the exposure time with the same soman vapor concentration. This increase in dose implies a risk that the animal would not survive the exposure. Therefore it was decided to pretreat the animals with HuBuChE. The dose of the HuBuChE was adjusted to 0.5 times the inhaled dose.

Table 25 Concentrations of C(+P(-)- and C(-)P(-)-soman (ng/mL) and BuChE (nM) in blood of anesthetized, atropinized and HuBuChE (27 nmol) - pretreated marmosets after a 5-min nose-only exposure to 50 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air.

Animal #	MS 5, 440 g			MS 6, 380 g			MS 7, 350 g			MS 8, 340g		
Time (min)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)	C(+)P(-)-soman	C(-)P(-)-soman	BuChE (nM)
0	< LOD	< LOD	118	< LOD	< LOD	223	< LOD	< LOD	120	< LOD	< LOD	n.m.
5.5	< LOD	< LOD		< LOD	< LOD	151	0.348	0.809	32	dead	dead	n.m.
8	< LOD	< LOD	65	< LOD	< LOD	130	0.318	0.617	26			
10	< LOD	< LOD	71	< LOD	< LOD	131	0.239	0.269	24			
20	< LOD	< LOD		< LOD	< LOD	127	< LOD	< LOD	27			
40	< LOD	< LOD	69	< LOD	< LOD	122	< LOD	< LOD	26			
60	< LOD	< LOD	52	< LOD	< LOD	127	< LOD	< LOD	22			
AUC ($ng.min.mL^{-1}$)							2.4	3.65				
F-react ($ng.mL^{-1}$)		2.96			2.515			failed			2.86	

LOD = limit of detection (50 pg/mL), AUC is expressed in $ng.min.mL^{-1}$, F-react is expressed in ng soman/ mL plasma, n.m. = not measured

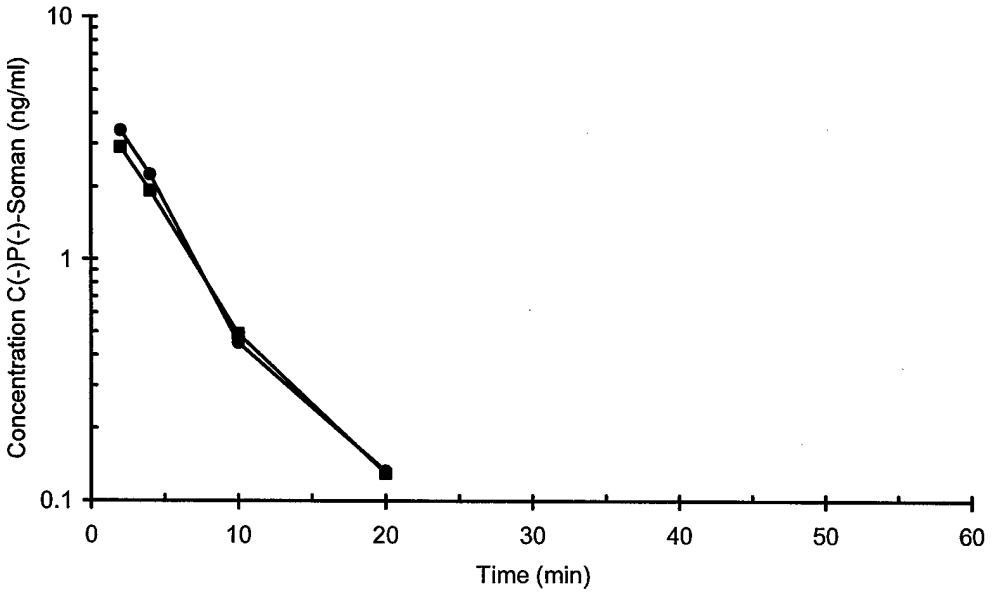

Figure 19 Concentration of C(±)P(-)-soman stereoisomers (ng/mL) in blood of an anesthetized and atropinized marmoset after a 2-min nose-only exposure to 50 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air. C(+)P(-)-soman (●), C(-)P(-)-soman (■).

Assuming a respiratory minute volume of 80 mL and a retention of 50%, the inhaled dose would be: 80 mL/min * 5 min * 50 $mg.m^{-3}$ * 50% = 10 µg ≡ 55 nmol. The required dose of HuBuChE would be 27 nmol.

Marmosets were injected with 27 nmol HuBuChE and were anesthetized and atropinized 16 h later. A carotid artery cannula was inserted and the animal was exposed for 5-min to 50 $mg.m^{-3}$ soman vapor. Blood samples were drawn after the exposure at 6, 8, 10, 20, 40 and 60 min. The volumes of the blood samples were 0.2 mL for time points 0 and 6 and 0.3 mL for the remaining time points. The results are shown in Table 25. Intact soman could only be measured in blood of animal 7. The concentration of BuChE in the corresponding blood sample was rather low. Soman could also be regenerated in the final blood sample. Soman was not detected in blood of animal 5 and 6. The residual BuChE concentration in blood was higher than in animal 7. Therefore it is questionable whether animal 5 and 6 inhaled the same dose as animal 7. Animal 8 died immediately after the exposure. It was possible to take only one blood sample that was used for the fluoride reactivation assay. Soman could be regenerated which is a verification that soman was inhaled. It was intended to perform more animal experiments with marmosets. However marmoset 9 and 10 died a few min after they were anesthetized. During these experiments some doubts raised about a possible interaction between enzyme, heparin and anesthetics. It was decided to discontinue these experiments, because this would sacrifice more marmosets without a reasonable chance to meaningful results.

The animals that survived the exposure showed no intact soman in blood (animal 7 excepted). Apparently the scavenger functioned well or the challenge was insufficient. Therefore it was decided to perform some additional experiments with marmosets that were not pretreated with

HuBuChE but to expose them to the same conditions (5 min, 50 $mg.m^{-3}$ soman vapor). Two animals were exposed, which both survived the exposure. Blood was sampled after the exposure according to the same schedule as described for the HuBuChE-pretreated animals. Table 26 shows the results. Intact soman was found in blood of both animals. Again the concentrations of C(+)P(-)-isomers were higher than those of the C(-)P(-)-isomers. The deviation between the two curves was too high to construct one averaged curve. AUCs were calculated for each curve by numerical integration. The deviation in AUC is not verified by the amount of regenerable soman. Apparently a small amount of soman is sufficient to saturate the regenerable binding sites. The data shows that the addition of scavenger was effective in reducing the concentration of intact soman.

Table 26 Concentrations of C(+)P(-)- and C(-)P(-)-soman stereoisomers (ng/mL) in blood of anesthetized and atropinized marmosets after a 5-min nose-only exposure to 50 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air.

Animal #	MS 3, 507 g		MS 4, 356 g	
Time	C(+)P(-)-soman	C(-)P(-)-soman	C(+)P(-)-soman	C(-)P(-)-soman
0				
6	0.52	0.42	5.03	3.58
8	0.27	0.20	3.18	2.33
10	0.29	0.09	2.24	1.81
20	< LOD	0.04	0.32	0.25
40	< LOD	< LOD	< LOD	< LOD
60	< LOD	< LOD	< LOD	< LOD
AUC ($ng.min.mL^{-1}$)	4.6	2.1	30.6	23.6
F-react ($ng.mL^{-1}$)		3.12		3.28

LOD = limit of detection (50 pg/mL), F-react is expressed in ng soman/ mL plasma

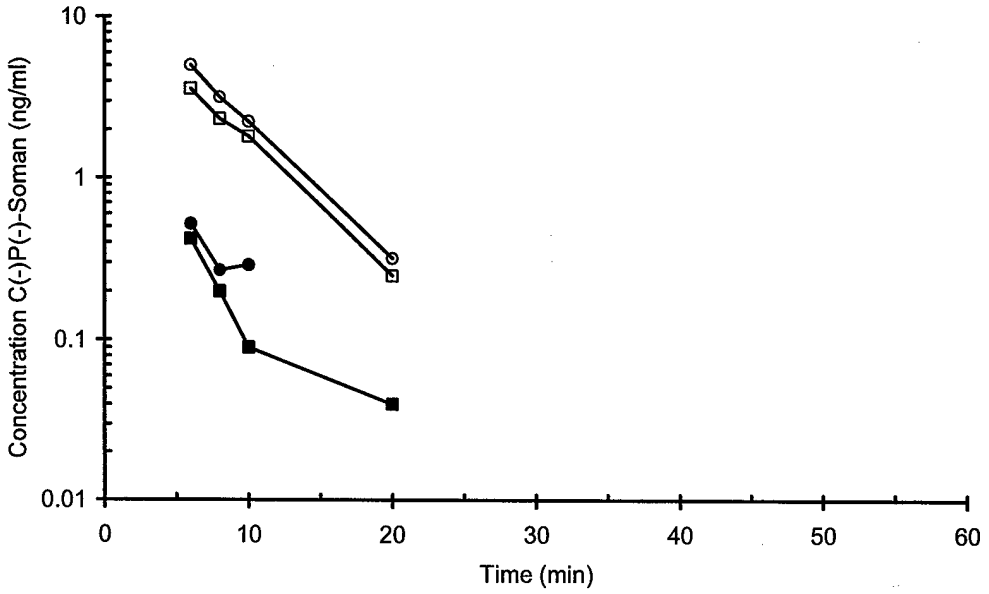

Figure 20 Concentrations of C(±)P(-)-soman stereoisomers (ng/mL) in blood of anesthetized and atropinized marmosets after a 5-min nose-only exposure to 50 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air. C(+)P(-)-soman MS 3 (•), C(-)P(-)-soman MS3 (■), C(+)P(-)-soman MS 4 (○), C(-)P(-)-soman MS 4 (□)

III.11 DETERMINATION OF THE RATE CONSTANT OF INHIBITION OF HuBuChE BY THE STEREOISOMERS OF THE NERVE AGENTS C(±)P(±)-SOMAN, (±)-SARIN AND (±)-VX (T.O.s 14-17)

The concentration of HuBuChE can be expressed as units per ml (U/mL) in which 1 U hydrolyses 1 µmol of butyrylthiocholine in 50 mM phosphate buffer per min at pH 8.0 and 25 °C. The concentration of a solution of HuBuChE expressed in U/mL was evaluated after determining the BuChE activity under these conditions, using the molar extinction coefficient of 2-nitro-5-mercapto-benzoic acid, i.e., the reaction product of which the extinction is measured in the activity assay. The molar extinction coefficient of 2-nitro-5-mercapto-benzoic acid was determined to be $1.396 * 10^4$. It is known from literature (Grunwald *et al.*, 1997) that 1 nmol of HuBuChE active sites corresponds with 60 units (U) of the enzyme. Hence, the molar concentrations of the HuBuChE active sites could subsequently be calculated from the enzyme concentration expressed as U/mL. This molar concentration of the enzyme should be known, in addition to that of the inhibitor in order to determine the rate constants of inhibition for the potent inhibitors (±)-sarin, (±)-VX and C(±)P(±)-soman.

The results obtained from reactions of the racemic mixtures of (±)-sarin and (±)-VX with HuBuChE did not completely obey the kinetics for a reaction of the enzyme with the two stereoisomers of the nerve agents reacting at the same rate, nor kinetics for reaction of the enzyme with only one stereoisomer due to high stereoselectivity. It was concluded from these results that HuBuChE shows a relatively low selectivity for the stereoisomers of these two organophosphates.

Values for the rate constants of inhibition of HuBuChE by the two stereoisomers of (±)-sarin and (±)-VX were obtained from a series of experiments in which inhibition of the enzyme by the racemic mixture of the organophosphate was followed with time. In these experiments, enzyme and inhibitor concentrations were only slightly different. The rate constants for the two parallel reactions proceeding with the two stereoisomers were evaluated by introducing a parameter θ, defined as $\theta = \int [E]\, dt$, in which [E] and t are enzyme concentration and reaction time, respectively, analogous to a procedure described by French (1950) (see Appendix 1). The calculated rate constants are presented in Tables 27 and 28.

Table 27 Rate constants for inhibition (k, $M^{-1}.min^{-1}$) of HuBuChE by the two stereoisomers of (±)-sarin at pH 7.4 and 38 °C; the initial concentrations of enzyme and inhibitor in the inhibition reactions are also shown.

Run	HuBuChE (nM)	Sarin (nM)	k_1 ($M^{-1}.min^{-1}$)	k_2 ($M^{-1}.min^{-1}$)	k_1/k_2
1	12.6	12.0	$4.1 * 10^7$	$6.1 * 10^6$	6.7
2	11.8	10.5	$4.2 * 10^7$	$4.4 * 10^6$	9.7
3	12.8	10.5	$4.2 * 10^7$	$6.1 * 10^6$	7.0
Mean			$(4.2 \pm 0.1) * 10^7$	$(5.5 \pm 1.0) * 10^6$	7.6

k_1 pertains to (-)-sarin, k_2 pertains to (+)-sarin

Table 28 Rate constants for inhibition (k, $M^{-1}.min^{-1}$) of HuBuChE by the two stereoisomers of (±)-VX at pH 7.4 and 38 °C; the initial concentrations of enzyme and inhibitor in the inhibition reactions are also shown.

Run	HuBuChE (nM)	VX (nM)	k_1 ($M^{-1}.min^{-1}$)	k_2 ($M^{-1}.min^{-1}$)	k_1/k_2
1	11.0	21	$0.95 * 10^7$	$3.4 * 10^6$	2.8
2	11.1	18	$1.45 * 10^7$	$1.2 * 10^6$	12
3	10.4	10.5	$1.27 * 10^7$	$2.7 * 10^6$	4.7
4	10.6	9.7	$1.39 * 10^7$	$3.5 * 10^6$	4.0
5	5.7	9.7	$1.34 * 10^7$	$3.5 * 10^6$	3.8
Mean			$(1.3 \pm 0.2) * 10^7$	$(2.8 \pm 0.9) * 10^6$	4.4

k_1 pertains to (-)-VX, k_2 pertains to (+)-VX

The rate constants of inhibition of HuBuChE by the four stereoisomers of C(±)P(±)-soman cannot be deduced in the same way as it was accomplished with (±)-sarin and (±)-VX. Therefore, the individual isomers of soman were isolated from C(+)P(±)-soman and C(-)P(±)-soman in the usual manner by treatment with either α-chymotrypsin or rabbit serum (Benschop *et al.*, 1984).

The inhibition reactions were performed under first-order conditions with respect to both enzyme and soman stereoisomer, except for inhibition by C(+)P(+)-soman. This isomer was used in a sufficiently large excess over the enzyme in order to justify description of the reaction as a pseudo first-order process. The results obtained for the four stereoisomers are presented in Tables 29-32.

Table 29 Rate constants for inhibition (k, $M^{-1}.min^{-1}$) of HuBuChE by C(-)P(+)-soman at pH 7.4 and 38 °C; the initial concentrations of enzyme and inhibitor in the inhibition reactions are also shown.

Run	HuBuChE (nM)	C(-)P(+)-soman (nM)	k ($M^{-1}.min^{-1}$)
1	3.57	13.0	$7.1 * 10^6$
2	3.75	11.8	$7.3 * 10^6$
3	3.57	10.1	$6.7 * 10^6$
4	3.55	8.8	$7.3 * 10^6$
5	3.57	7.1	$8.3 * 10^6$
Mean			$(7.4 \pm 0.6) * 10^6$

Table 30 Rate constants for inhibition (k, $M^{-1}.min^{-1}$) of HuBuChE by C(+)P(+)-soman at pH 7.4 and 38 °C; the initial concentrations of enzyme and inhibitor in the inhibition reactions are also shown.

Run	HuBuChE (nM)	C(+)P(+)-soman (nM)	k ($M^{-1}.min^{-1}$)
1	2.85	71	$2.2 * 10^6$
2	3.55	64	$2.3 * 10^6$
3	3.45	58	$2.0 * 10^6$
4	3.63	49	$2.3 * 10^6$
Mean			$(2.1 \pm 0.2) * 10^6$

Table 31 Rate constants for inhibition (k, $M^{-1}.min^{-1}$) of HuBuChE by C(+)P(-)-soman at pH 7.4 and 38 °C; the initial concentrations of enzyme and inhibitor in the inhibition reactions are also shown.

Run	HuBuChE (nM)	C(+)P(-)-soman (nM)	k ($M^{-1}.min^{-1}$)
1	1.01	0.96	$3.5 * 10^8$
2	0.98	0.96	$3.6 * 10^8$
3	1.11	0.95	$3.8 * 10^8$
Mean			$(3.6 \pm 0.2) * 10^8$

Table 32 Rate constants for inhibition (k, $M^{-1}.min^{-1}$) of HuBuChE by C(-)P(-)-soman at pH 7.4 and 38 °C; the initial concentrations of enzyme and inhibitor in the inhibition reactions are also shown.

Run	HuBuChE (nM)	C(-)P(-)-soman (nM)	k ($M^{-1}.min^{-1}$)
1	10.7	26.7	$1.26 * 10^7$
2	11.6	22.4	$1.31 * 10^7$
3	8.6	21.1	$1.06 * 10^7$
4	12.3	19.9	$1.20 * 10^7$
Mean			$(1.2 \pm 0.1) * 10^7$

III.12 BINDING IN EXTRAVASCULAR COMPARTMENTS (T.O. 18-20)

In earlier sections it was shown that about 25 % of the i.m. administered dose of HuBuChE reaches the blood. Therefore it is interesting to see what the fate of the administered dose of HuBuChE is. Guinea pigs were i.m. injected with a dose of HuBuChE. At 24 h after administration the animals were euthanized and blood was collected by means of a heart punction. Next the organs (liver, lung, heart, kidney, brain) were removed. The organs were weighed and homogenates (25% in saline) were prepared. The BuChE activity in the homogenate was determined with the Ellman assay. Next the homogenates were centrifuged and the supernatant was pipetted from the residue. The residue was washed with fresh saline and centrifuged again. The supernatants were pooled and used for the determination of BuChE activity. The recovery of the BuChE in the supernatants appeared to be 90-100% for all homogenates. It was decided to perform all BuChE measurements with the supernatants, in order to avoid interference of the turbid homogenates with the spectrophotometric determination. Table 33 shows the results of the BuChE activity measurements.

Table 33 BuChE activity (U) in blood and organs of HuBuChE-treated guinea pigs. Animal 1 was not pretreated with HuBuChE

Animal	GP 1, 948 g		GP 2, 407 g		GP 3, 600 g		GP 4, 470 g		GP 5, 620 g	
Dose (U)	0		4626		2970		5421		5421	
	Weight (g)	Units	Weight (g)	Units	Weight (g)	Units	Weight (g)	Units	Weight (g)	Units
blood	56	142	24	1147	36	1004	28	1771	37	1664
liver	47	239	20	208	27	249	26	385	38	279
lung	3.8	4.4	2.9	41	3.0	14	2.9	29	3.2	58
heart	4	2.5	1.8	25	2.0	7.7	1.7	17	3.2	55
kidney	7.3	5.2	3.6	52	4.4	11	4	54	4.7	62
brain	3.7	2.6	3.1	6.2	3.6	4.6	3.8	5.3	3.9	11

It is difficult to compare the results of the various animals with each other, because the animals differed in weight. In Table 34 the results are normalized a body weight of 1 kg.

Table 34 BuChE activity (U) in blood and organs of HuBuChE treated guinea pigs. Activity and weight of organs are linearly normalized to body weight of 1000 g.

Animal	1		2		3		4		5	
Dose	0		11366		4950		11534		8743	
	Weight (g)	Units	Weight (g)	Units	Weight (g)	Units	Weight (g)	Units	Weight (g)	Units
blood	60	150	60	2818	60	1673	60	3768	60	2683
liver	49	252	49	511	45	415	55	819	61	450
lung	4	4.64	7.12	100	5	23.3	6	62	5.1	94
heart	4.2	2.64	4.42	61.4	3.3	12.8	4	36	5.1	88
kidney	7.7	5.48	8.84	127	7.3	18.3	9	115	7.5	100
brain	3.9	2.74	7.6	15.2	6	7.6	8	11	6.2	18

It is clear that the BuChE activity in the tissue homogenates of the HuBuChE-pretreated animals is higher than in the tissue homogenates of the naive animal. However it must be considered that the homogenates contain a significant amount of blood, which contains a high concentration of BuChE. The amount of blood in tissue of guinea pigs has been measured by Bosse and

Wasserman (1970). They reported percentages of blood in liver (15%), lung (20%), heart (12%), kidney (20%) and brain (2%). Using these values the amount of BuChE contributed by blood in tissue homogenate can be calculated. By subtracting the BuChE activity contributed by blood from the total BuChE activity in the homogenate, the BuChE activity in the extravascular compartment of the particular tissue is obtained. Table 35 shows the estimated activity of BuChE in the extravascular compartments.

Table 35 Estimated BuChE activity in extravascular compartment of organs in HuBuChE pretreated guinea pigs. Animal 1 was not pretreated with HuBuChE.

Animal	1		2		3		4		5	
Dose		0		11366		4950		11534		8743
	Weight (g)	Units	Weight (g)	Units	Weight (g)	Units	Weight (g)	Units	Weight (g)	Units
blood	60	149	60	2818	60	1673	60	3768	60	2684
liver	49	233	49	158	45	226	55	294	61	36
lung	4	2.6	7.12	32.6	5	- 4.6	6	-16	5.1	47
heart	4.2	1.4	4.42	36.1	3.3	1.7	4	8.7	5.1	60
kidney	7.7	1.6	8.84	43.2	7.3	-22.6	9	7.2	7.5	31
brain	3.9	2.5	7.6	8.0	6	4.3	8	1.0	6.2	12

The values in Table 35 are only very rough estimations because the correction factor (BuChE in blood) has a large contribution to the total activity of the homogenate, which is in many cases not much higher. Sometimes even negative values are obtained, which means that the concentration of BuChE in the extravascular compartments is not very high. The amount of BuChE in the liver of HuBuChE-pretreated animals is not higher than in the liver of a naive animal, which indicates that the extravascular compartments of the liver (natural source of BuChE) are not further loaded with HuBuChe that was i.m. administered. On the other hand the concentration of HuBuChE in the extravascular compartments of the other organs were higher than in the naive animal, which means that these compartments were loaded with HuBuChE that was administered.

Table 36 Relative distribution of BuChE activity over blood and organs in HuBuChE-pretreated guinea pigs. BuChE activity in organs is corrected for blood contents. Animal 1 was not pretreated with HuBuChE and is not taken into account in the calculation of the average distribution.

Animal #	1	2	3	4	5	Mean (2-5) ± SEM
Dose (U/kg)		11366	4950	11534	8743	
	%	%	%	%	%	%
blood	38.1	24.8	33.8	32.7	30.7	30.5 ± 2.0
liver	59.4	1.4	4.6	2.6	0.4	2.2 ± 0.9
lung	0.1	0.3	-0.1	-0.1	0.5	0.1 ± 0.2
heart	0.5	0.3	0.0	0.1	0.7	0.3 ± 0.2
kidney	1.2	0.4	-0.5	0.1	0.4	0.1 ± 0.2
brain	0.7	0.1	0.1	0.0	0.1	0.1 ± 0.03
unknown	-	72.8	62.0	64.8	67.1	66.7 ± 2.3

The relative distribution of BuChE over blood and organs can be averaged from results for animals 2-5. The distribution is shown in Table 36 and Figure 21. About 30 % of the BuChE activity is found in blood, which is in reasonable agreement with most of the toxicokinetic and

pharmacokinetic experiments. The liver (source of native BuChE) contains also a significant amount of BuChE. The amount of BuChE in the extravascular compartments in the other organs is negligible. The fate of the most of the administered BuChE is still undiscovered, estimated at 67% of the total amount of administered HuBuChE.

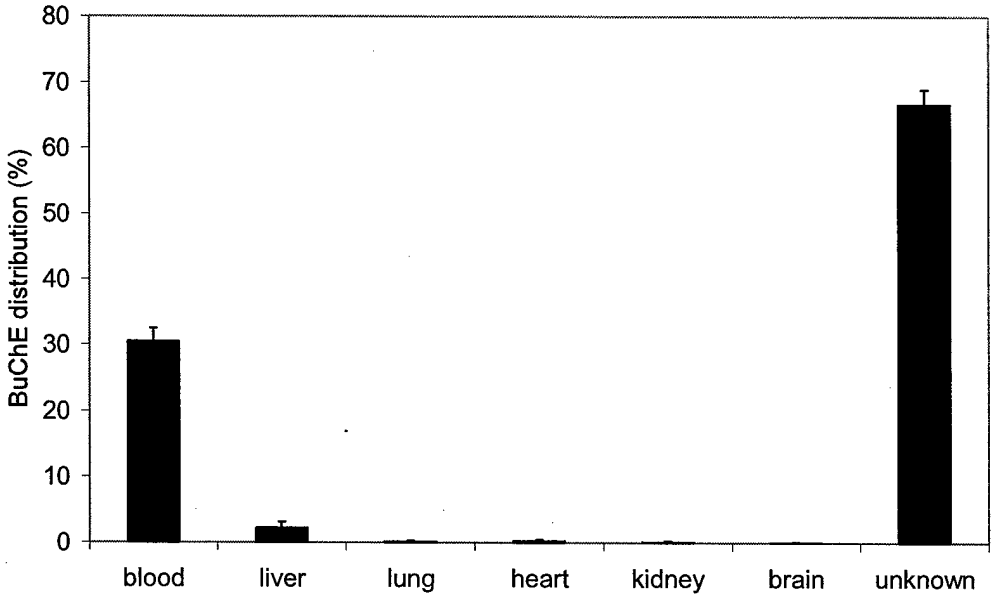

Figure 21 Distribution of BuChE in guinea pigs at 24 h after i.m. administration of HuBuChE

If the amount of BuChE that cannot be traced is not taken into account, it is possible to compare the BuChE distribution before and after HuBuChE administration. Table 37 and Figure 22 show the distribution of BuChE over blood and the measured organs. The black bar indicates BuChE in a naive animal, the grey bars show the average BuChE distribution in HuBuChE-treated animals. Figure 22 shows clearly that the main part of BuChE in a non-pretreated animal is present in the liver. In a HuBuChE-pretreated animal the main part of HuBuChE is present in blood, which is advantageous for scavenging.

Table 37 Distribution of BuChE activity over blood and measured organs in HuBuChE-pretreated guinea pigs. BuChE activity in organs is corrected for blood contents. Animal 1 was not pretreated with HuBuChE and is not taken into account in the calculation of the average distribution.

Animal	1	2	3	4	5	Mean (2-5) ± SEM
Dose (U/kg)		11366	4950	11534	8743	
	%	%	%	%	%	%
blood	38.1	87.2	86.0	88.8	90.2	88.1 ± 0.9
liver	59.4	4.9	11.7	6.9	1.2	6.2 ± 2.2
lung	0.1	2.7	0.7	1.0	2.9	1.8 ± 0.6
heart	0.5	1.7	0.6	0.7	2.6	1.4 ± 0.5
kidney	1.2	3.2	0.7	2.3	2.5	2.2 ± 0.5
brain	0.7	0.4	0.4	0.2	0.5	0.4 ± 0.1

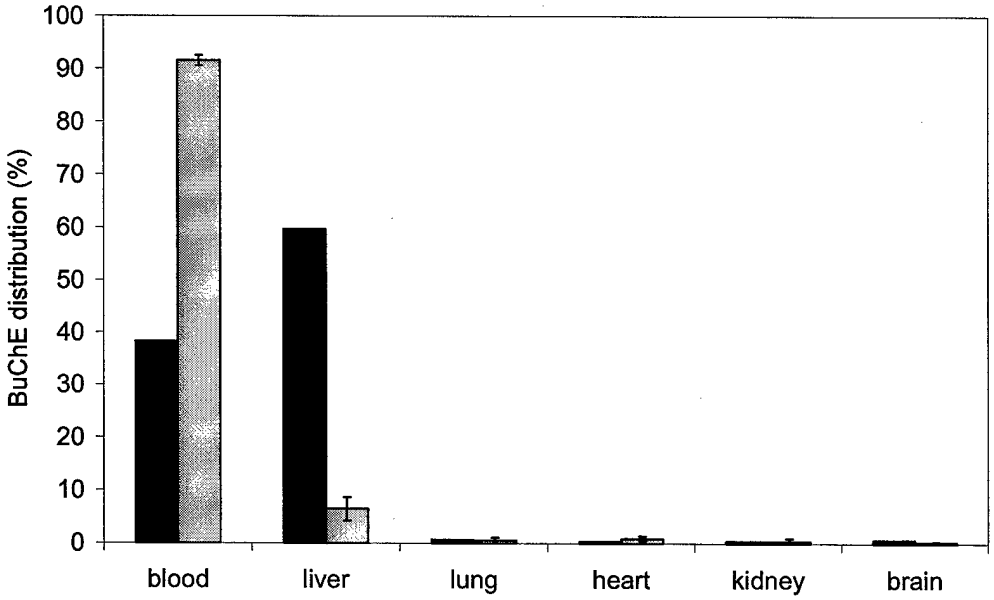

Figure 22 Relative distribution of BuChE in native guinea pig (black) and HuBuChE pretreated guinea pigs (grey) at 24 h after pretreatment.

In another experiment a guinea pig (500 g) was i.m. injected with HuBuChE (90 nmol). At 24 h after the administration, the animal was anesthetized, atropinized and artificially ventilated. The animal received a dose of radioactively labeled ^{14}C-[Methyl]-soman (55 mCi/mmol) via the vena jugularis (intravenous injection) at a dose of 110 µg/kg which corresponds with 4 LD50. After 90 min the guinea pig was euthanized. Blood was collected and the organs (liver, lung, heart, kidney and brain) were removed. Homogenates were prepared and further processed as described in section II.2.18-20. The remaining BuChE activity in blood and in the supernatant of each homogenate was determined according to the Ellman assay (see Table 40). The radioactivity in blood and in the supernatant was determined using liquid scintillation counting. Table 38 shows the results. The amount of recovered radioactivity was 3.8e7 dpm, which is slightly more than the administered dose. The amount of recovered radioactivity corresponds with 309 nmol soman or its hydrolysis products or adducts. If the radioactivity in the homogenates is corrected for the blood contents in the particular tissues, the recovery of radioactivity is 95%. Table 38 shows that blood, liver, kidney and lung are the most important targets for soman be it intact, hydrolyzed or adducted. The distribution of radioactivity is in rather good agreement with the data found in earlier work (DAMD17-87-F-7015) except for the values in blood. In earlier work about 14% of the radioactivity was recovered in blood, while the recovery in this study was more than 55%.

Table 38 Distribution of radioactivity over blood and organs in a guinea pig that was pretreated with HuBuChE (90 nmol) and i.v. injected with ^{14}C-soman (Soman 54 µg ≡ 295 nmol ≡ 3.6e7 dpm)

	Homogenates				Corrected for blood content in organs			
tissue	dpm	µg Soman	nmol Soman	(%)	dpm	µg Soman	nmol Soman	(%)
blood	2.0e7	29.8	164	55.5	2.0e7	29.8	164	55.5
liver	6.3e6	9.4	51.7	17.5	3.9e6	5.91	32.4	11.0
lung	4.0e6	5.9	32.5	11.1	3.5e6	5.27	29.0	9.8
heart	3.7e5	0.56	3.1	1.03	2.2e5	0.33	1.83	0.61
kidney	7.2e6	10.6	58.6	20.0	6.5e6	9.72	53.4	18.1
brain	9.2e4	0.14	0.75	0.26	4.5e4	0.068	0.376	0.127
total	3.8e7	56.4	309	106	3.4e7	51.1	281	95.2

It was attempted to differentiate the fate of soman into hydrolyzed and BuChE-bound soman. The amount of hydrolyzed soman was determined by extracting the hydrolysis product pinacolyl methylphosphonic acid (PMPA) after acidification with a mixture of toluene:butanol. The radioactivity in the organic layer is a measure for the amount of PMPA. The amount of soman covalently bound to BuChE was determined by means of procainamide affinity chromatography (see section II.2.20). Samples were eluted on a procainamide gel filled column which shows affinity for BuChE. Table 39 shows the differentiation of the radioactivity between PMPA and BuChE bound soman. The total recovered radioactivity corresponds with 2.8e7 dpm, which is 77% of the total amount of radioactivity that was administered. The amount of radioactivity in the brain was too low to be detected after procainamide affinity chromatography. From Tables 35 and 36 it could be concluded that the extravascular compartments were only slightly loaded with HuBuChE. The data in Table 39 verifies this finding, the amount of soman that was bound to BuChE in extravascular compartments was very low compared to the amount BuChE bound soman that was found in blood. Table 39 shows also that the hydrolysis product of soman is present in the extravascular compartments of liver, kidney and lung.

Table 39 Distribution of hydrolyzed and BuChE-bound soman over tissues and blood in a HuBuChE-pretreated guinea pig. (Soman 54 µg≡ 295 nmol ≡ 3.6e7 dpm)

		Homogenates			Corrected for blood content in organs		
		µg	nmol	dpm	µg	nmol	dpm
blood	hydrolyzed	11.6	63.7	7.8e6	11.6	63.7	7.8e6
	bonded	7.3	40.1	4.9e6	7.3	40.1	4.9e6
	sum	18.9	104	1.3e7	18.9	104	1.3e7
liver	hydrolyzed	8.6	47.6	5.8e6	7.3	40.1	4.9e6
	bonded	0.59	3.26	4.0e5	-0.3	-1.4	-1.7e5
	sum	9.2	50.9	6.2e6	7.0	38.7	4.7e6
lung	hydrolyzed	2.12	11.7	1.43e6	1.86	10.2	1.3e6
	bonded	0.42	2.30	2.82e5	0.25	1.4	1.7e5
	sum	2.55	14.0	1.7e6	2.11	11.6	1.5e6
heart	hydrolyzed	0.22	1.22	1.49e5	0.14	0.75	9.1e4
	bonded	0.27	1.48	1.81e5	0.22	1.19	1.5e5
	sum	0.49	2.70	3.3e5	0.36	1.94	2.1e5
kidney	hydrolyzed	14.2	78.1	9.5e6	13.8	75.7	9.2e6
	bonded	0.23	1.29	1.6e5	-0.01	-0.05	-5.8e3
	sum	14.5	79.4	9.7e6	13.8	75.7	2.5e5
brain	hydrolyzed	0.04	0.23	2.9e4	0.02	0.09	1.1e4
	bonded	< LOD	< LOD	< LOD	< LOD	< LOD	<LOD
	sum	0.04	0.23	2.9e4	0.02	0.09	1.8e4
Total		45.7	251	3.06e7	42.2	232	2.8e7

Finally it was attempted to construct a recovery sheet of the amount of BuChE. The amount of free BuChE in blood and homogenates was determined with the Ellman assay. The amount of inhibited HuBuChE was determined based on the amount of radioactivity that was recovered after isolation of BuChE using the procainamide affinity column. Table 40 shows the results. The total amount of BuChE that could be recovered was 44 nmol (49%) which is slightly higher than found in earlier experiments (see Table 36).

Table 40 Fate of BuChE in blood and organs of a HuBuChE-pretreated guinea pig at 90 min after intravenous injection of soman (55 µg). (HuBuChE: 5400 Units ≡ 90 nmol)

		Homogenates			Corrected for blood content in organs		
		Units	nmol	dpm	Units	nmol	dpm
blood	free	109	2		109	1.8	
	inhibited		40.1	4.9e6		40.1	4.9e6
	sum		42.1			42.1	
liver	free	54	0.9		41.3	0.69	
	inhibited		3.26	4.0e5		-1.4	-1.7e5
	sum		4.16			-0.71	
lung	free	3.5	0.06		0.98	0.016	
	inhibited		2.30	2.82e5		1.39	1.7e5
	sum		2.36			1.40	
heart	free	1.5	0.02		0.69	0.011	
	inhibited		1.48	1.81e5		1.19	1.5e5
	sum		1.50			1.20	
kidney	free	4.3	0.07		0.61	0.011	
	inhibited		1.29	1.6e5		-0.05	-5.8e3
	sum		1.36			-0.039	
brain	free	0.3	0.005		0.05	0.0008	
	inhibited	-	-	-		-	-
	sum		0.005			0.0008	
total			51.5			43.9	

It is clear that soman or its hydrolysis product reached all organs. The amount of BuChE in the liver remained quite high (82 U / liver, BW =1000 g) compared to the values in of naive guinea pig or HuBuChE pretreated guinea pig (see Table 35). The other organs contain only very low amounts of BuChE.

III.13 PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELLING (T.O. 21)

A Physiologically Based PharmacoKinetic (PBPK) model describing the toxicokinetics of the two most toxic isomers of soman in the guinea pig was developed and programmed using Labview. Labview™ (National Instruments, Austin, TX, USA) is a graphical programming language mostly used for data-acquisition and data processing, but it can be used for modeling. Langenberg *et al* (1997) also published a PBPK model for soman which consisted of ten different compartments. The present PBPK model is simplified to four compartments: blood, richly perfused, poorly perfused and liver. The compartments, richly, poorly and liver are fed by blood, while in blood itself reactions take place as well, thus blood is also compartment in the model.

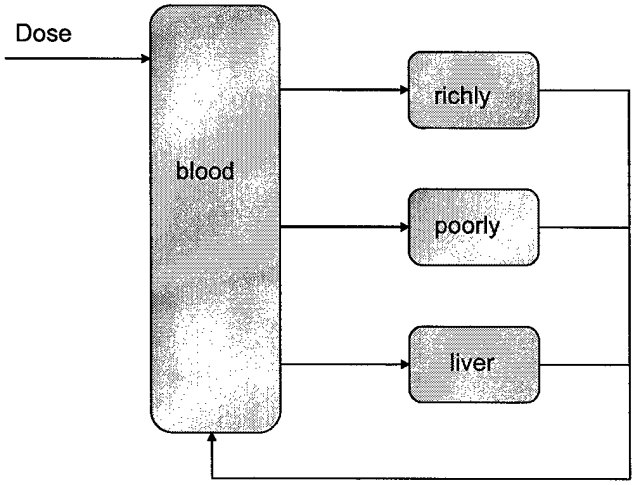

Figure 23 Schematic outline of a physiologically based pharmacokinetic model for the toxicokinetics of soman

Reactions taking place in each compartment are binding to AChE, BuChE, CaE and enzymatic hydrolysis. Each of these reactions is characterized by the reaction rate equation. The concentration of the toxicant and enzymes can be calculated by numerical integration of the mass balance that can be proposed for each compound. Basically the equations in the four compartments are the same, but the coefficients (biological parameters) are specific for each compartment.
In each compartment the following mass balances must be solved:

$$\frac{d[GD]}{dt} = r_{GD}$$

$$\frac{d[AChE]}{dt} = r_{AChE}$$

$$\frac{d[BuChE]}{dt} = r_{BuChE}$$

$$\frac{d[CaE]}{dt} = r_{CaE}$$

$$r_{AChE} = k_{AChE-GD} * [AChE] * [GD]$$

$$r_{BuChE} = k_{BuChE-GD} * [BuChE] * [GD]$$

$$r_{CaE} = k_{CaE-GD} * [CaE] * [GD]$$

$$r_{hydrol} = k_{hydrol} * [GD]$$

$$r_{GD} = Q * (c_{in} / P - c_{out} / P) - r_{AChE} - r_{BuChE} - r_{CaE} - r_{hydrol}$$

in which [GD] is concentration soman (mol.l^{-1}), [AChE] is concentration AChE (mol.l^{-1}), [BuChE] is concentration BuChE (mol.l^{-1}), [CaE] is concentration CaE (mol.l^{-1}), r_{GD} is reaction rate of soman (mol.l^{-1}.s^{-1})., r_{AChE} is reaction rate AChE (mol.l^{-1}.s^{-1}), r_{BuChE} is reaction rate BuChE (mol.l^{-1}.s^{-1}, r_{CaE} is reaction rate CaE (mol.l^{-1}.s^{-1}), r_{hydrol} is rate of enzymatic hydrolysis of GD (mol.l^{-1}.s^{-1}), $k_{AChE-GD}$ is binding constant between AChE and GD (M^{-1}.s^{-1}), $k_{AChE-GD}$ is binding constant between AChE and GD (M^{-1}.s^{-1}), $k_{BuChE-GD}$ is binding constant between BuChE and GD (M^{-1}.s^{-1}), k_{CaE-GD} is binding constant between, CaE and GD (M^{-1}.s^{-1}), k_{hydrol} is constant for enzymatic hydrolysis of C(±)P(-)-soman (s^{-1}), Q is blood flow (mL.s^{-1}), C_{in} concentration soman entering compartment (mol.l^{-1}), C_{out} is concentration soman leaving compartment (mol.l^{-1}), P is the partition coefficient tissue-blood.

In fact the number of reactions is two times higher, because the reactions of soman can be doubled for C(+)P(-) and C(-)P(+)-soman. The reaction of the P(+)-isomers are not taken into account. These isomers are rapidly eliminated by enzymatic hydrolysis.

Table 41 shows an overview of the binding constants and hydrolysis constants of soman in the various compartments. The values were obtained from *in vitro* measurements (De Jong, 1993, 1996).

Table 41 Biochemical parameters of the guinea pig used in the PBPK model

Parameter	Dimension	Blood	Poorly perfused	Richly Perfused	Liver
k hydrol	s^{-1}	8.2e-4	8.2e-4	3e-3	1.14e-2
k (AChE-C(+)P(-)-GD)	M^{-1}.s^{-1}	2.2e6	2.2e6	2.2e6	2.2e6
k (AChE-C(-)P(-)-GD)	M^{-1}.s^{-1}	7.5e5	7.5e5	7.5e5	7.5e5
k (BuChE-C(+)P(-)-GD)	M^{-1}.s^{-1}	6.0e6	6.0e6	6.0e6	6.0e6
k (BuChE-C(-)P(-)-GD)	M^{-1}.s^{-1}	2.0e5	2e5	2e5	2e5
k (CaE-C(+)P(-)-GD)	M^{-1}.s^{-1}	2.5e5	4.1e4	2.5e5	4.1e4
k (CaE-C(-)P(-)-GD)	M^{-1}.s^{-1}	1.2e5	8.1e3	1.2e5	8.1e3
AChE	nM	9	1.6	4.5	6.3
BuChE	nM	25	10	12	85
CaE	nM	500	10	200	20000
V	mL	60	760	110	50
Q	mL/s	2.8	0.67	1.68	0.45
P	-		2.0	2.8	2.0

Figures 24-26 show the calculated concentration-time curves of various toxicokinetic experiments with guinea pigs. The data points represent the actual measurements that were performed. Figure 24 shows the predicted intravenous toxicokinetics in guinea pigs after i.v. injection of a dose of soman which corresponds with 6 LD50. The binding sites are completely inhibited after administration of such high dose of soman. The elimination of soman is mainly described by distribution over the organs and enzymatic hydrolysis. The enzymatic hydrolysis is not stereoselective with regard to the C(+)P(-)- and C(-)P(-) isomers and the concentration-time curves of the P(-)-isomers are almost identical. Figure 25 shows the predicted concentration-time course of the C(±)P(-) isomers after i.v. injection of a dose of soman corresponding with 2 LD50. The dose is 3 times lower which means that the binding sites are not completely occupied.

The elimination proceeds more stereoselectively with regard to the C(+)P(-) and C(-)P(-)-isomers.

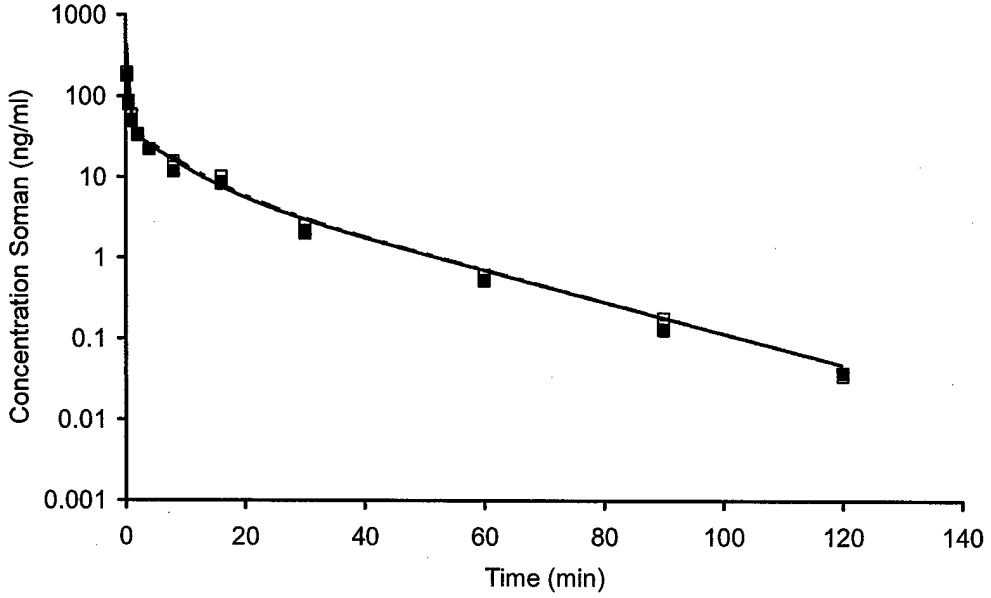

Figure 24 Predicted concentration (ng/mL) of C(+)P(-)-soman (dashed line) and C(-)P(-)-soman (solid line) in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 6 LD50 (165 µg/kg). Data points C(-)P(-)-soman (□) and C(+)P(-)-soman (■) from DAMD17-85-G-5004.

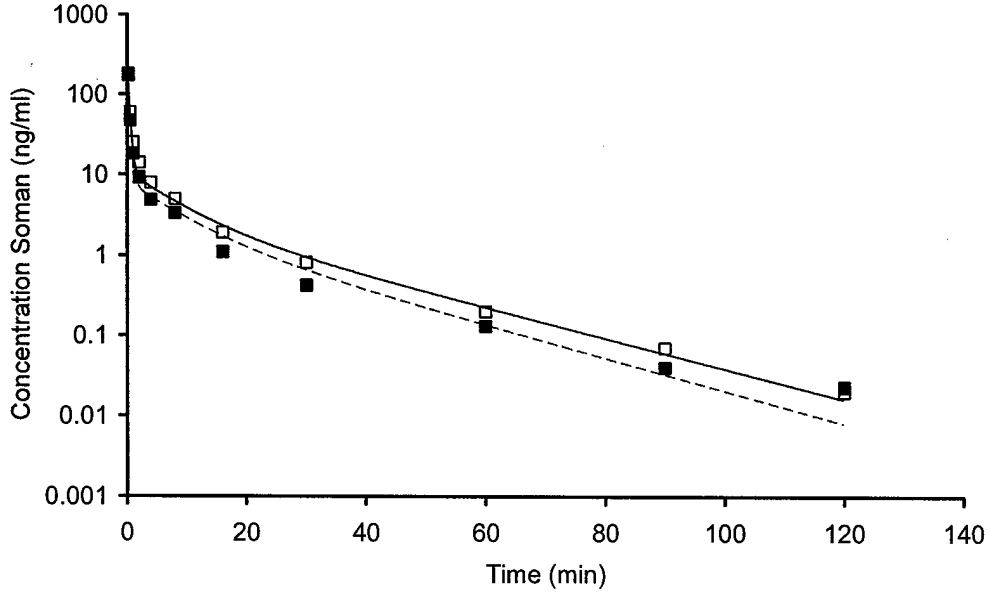

Figure 25 Predicted concentration (ng/mL) of C(+)P(-)-soman (dashed line) and C(-)P(-)-soman (solid line) in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg). Data points C(-)P(-)-soman (□) and C(+)P(-)-soman (■) from DAMD17-87-G-7015.

Figure 26 shows the predicted concentration-time course in blood of guinea pigs that were i.v. injected with soman corresponding with 0.8 LD50. The binding sites are not occupied by soman and the elimination of the soman isomers proceeds stereoselectively because of stereoselective binding of the C(±)P(-) isomers.

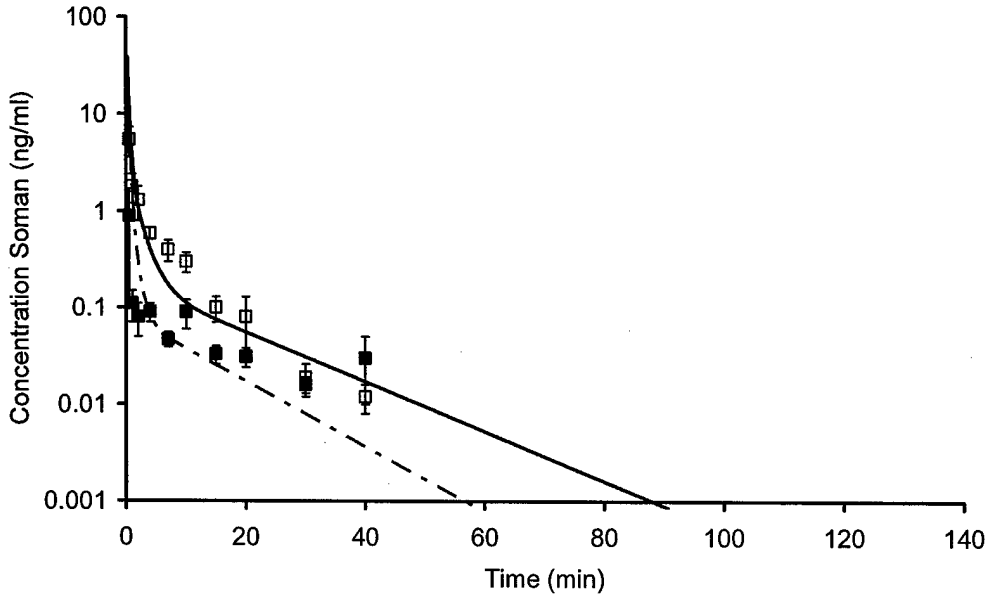

Figure 26 Predicted concentration (ng/mL) of C(+)P(-)-soman (dashed line) and C(-)P(-)-soman (solid line) in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 0.8 LD50 (22 µg/kg). Data points C(-)P(-)-soman (□) and C(+)P(-)-soman (■) from DAMD17-90-Z-0034.

In a next series of modeling experiments, the concentration of BuChE was increased in blood and the other three compartments. Table 42 shows the biochemical parameters in which the concentration of BuChE in the compartments was changed into values that were obtained after an i.m. injection of 200 nmol/kg HuBuChE (cf section III.12).

Table 42 Biochemical parameters of the guinea pig used in the PBPK model with modified concentrations of BuChE in the various compartments.

Parameter	Dimension	Blood	Poorly perfused	Richly Perfused	Liver
k hydrol	s^{-1}	8.2e-4	8.2e-4	3e-3	1.14e-2
k (AChE-C(+)P(-)-GD)	$M^{-1}.s^{-1}$	2.2e6	2.2e6	2.2e6	2.2e6
k (AChE-C(-)P(-)-GD)	$M^{-1}.s^{-1}$	7.5e5	7.5e5	7.5e5	7.5e5
k (BuChE-C(+)P(-)-GD)	$M^{-1}.s^{-1}$	6.0e6	6.0e6	6.0e6	6.0e6
k (BuChE-C(-)P(-)-GD)	$M^{-1}.s^{-1}$	2e5	2e5	2e5	2e5
k (CaE-C(+)P(-)-GD)	$M^{-1}.s^{-1}$	2.5e5	4.1e4	2.5e5	4.1e4
k (CaE-C(-)P(-)-GD)	$M^{-1}.s^{-1}$	1.2e5	8.1e3	1.2e5	8.1e3
AChE	nM	9	1.6	4.5	6.3
BuChE	nM	800	20	50	85
CaE	nM	500	10	200	20000
V	mL	60	760	110	50
Q	mL/s	2.8	0.67	1.68	0.45
P	-		2.0	2.8	2.0

Note: It is assumed that the binding constants of soman and BuChE of guinea pig and human are the same

Figure 27-29 show the same calculations, however with modified concentrations of BuChE in the four compartments. Figure 27 shows the predicted time course of soman isomers after i.v. injection of a dose soman corresponding with 6 LD50. The binding sites were occupied in naive animals and the addition of additional binding site (HuBuChE) is not sufficient to neutralize the dose of soman and thus will not result in a significant relative change in the toxicokinetics of soman.

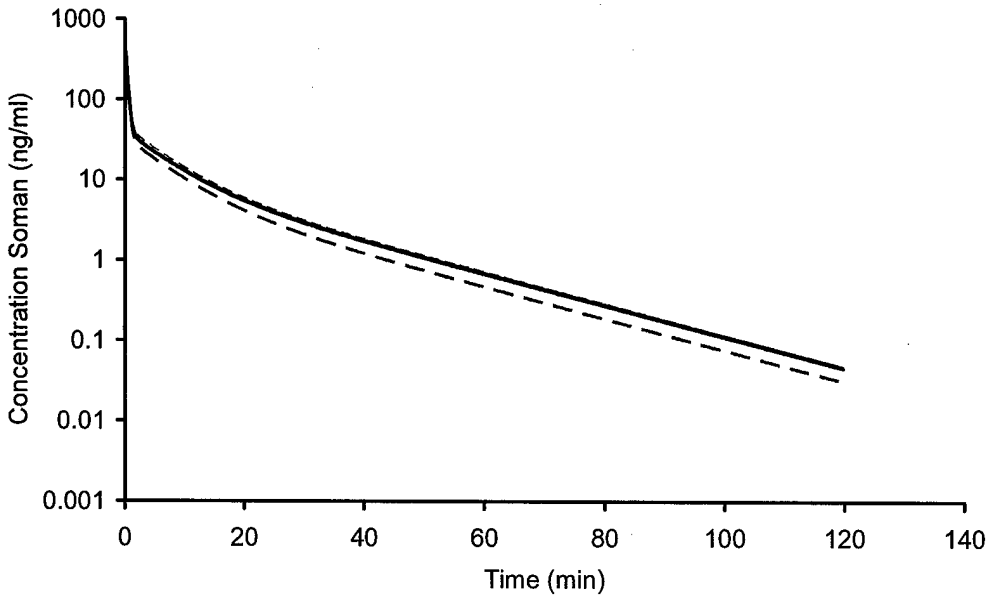

Figure 27 Predicted concentration (ng/mL) of C(+)P(-)-soman and C(-)P(-)-soman in blood of guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 6 LD50 (165 µg/kg). Solid lines naive animal, dashed lines HuBuChE-pretreated animal.

Figure 28 shows the predicted concentration-time course of the C(±)P(-)-isomers in naive and HuBuChE-pretreated animals after i.v. injection of a dose of soman corresponding with 2 LD50. In this case, the addition of HuBuChE is a significant factor in the elimination of soman isomers. The concentrations of both isomers are significantly reduced compared to the concentration in naive animals. It is also clear that the elimination of the C(+)P(-) isomer proceeds more effective than the C(-) isomer, which is explicable in view of the higher binding rate constant of BuChE with the C(+)P(-) isomer.

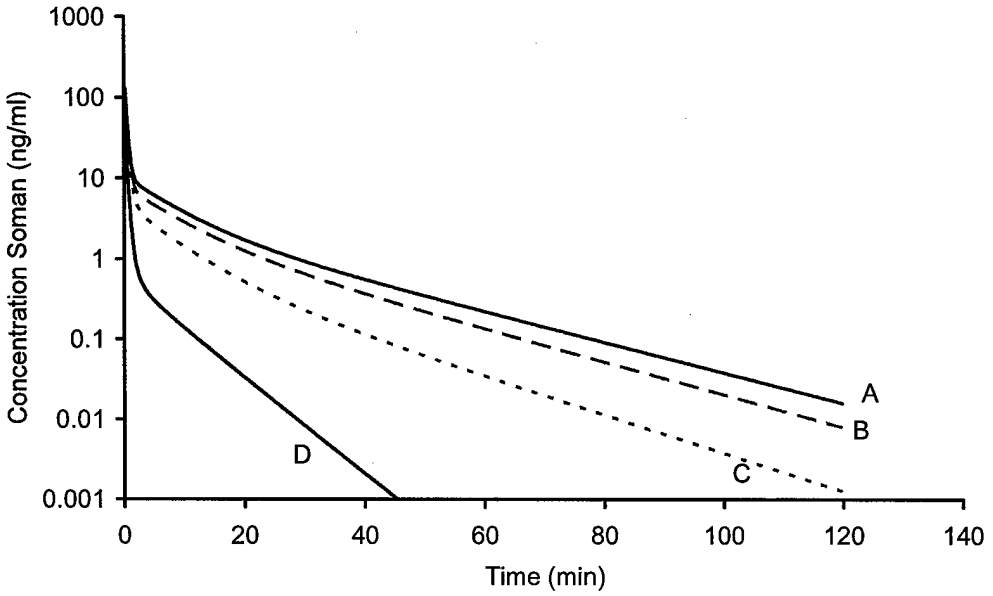

Figure 28 Calculated concentration (ng/mL) of C(+)P(-)-soman and C(-)P(-)-soman in blood of guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg). Naive animals: A: C(-)P(-)-soman, B: C(+)P(-)-soman; HuBuChE-pretreated animals: C: C(-)P(-)-soman, D: C(+)P(-)-soman

Figure 29 shows the predicted toxicokinetics of soman in naive and HuBuChE-pretreated animals after i.v. injection of a dose of soman corresponding with 0.8 LD50. The elimination of the C(±)P(-)-isomers proceeds with prominent stereoselectivity. The elimination of the C(+)P(-)-isomer proceeds extremely rapidly: it is completely eliminated within the first minute.

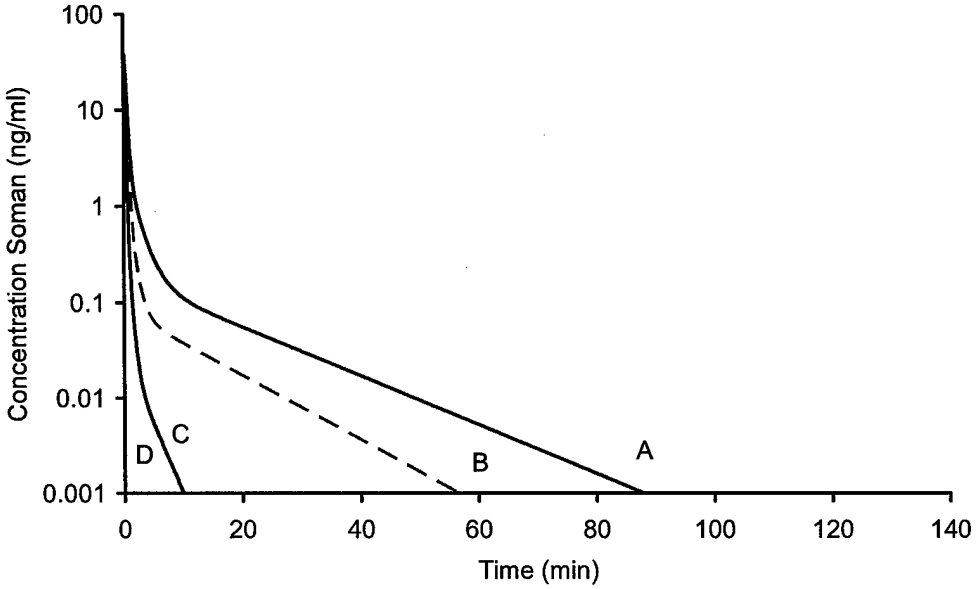

Figure 29 Predicted concentration (ng/mL) of C(+)P(-)-soman and C(-)P(-)-soman in blood of guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg). Naive animals: A: C(-)P(-)-soman, B: C(+)P(-)-soman; HuBuChE-pretreated animals: C: C(-)P(-)-soman, D: C(+)P(-)-soman.

IV DISCUSSION

IV. 1 Time course of HuBuChE concentrations in blood after i.m. administration

It is clear from Table 1 and Figure 1 that i.m. administration of HuBuChE leads to an important increase in the BuChE activity that can be measured in blood. The BuChE activity gradually builds up after i.m. administration of HuBuChE, reaches a maximum, and then decreases again. Table 1 and Figure 1 showed that the maximum concentration in blood of guinea pigs was found in the blood sample that was taken at 24 h after the i.m. injection of the enzyme. These values of Tmax were in agreement with results obtained by other researchers (Allon *et al,* 1998, Lenz, 2004). For the subsequent toxicokinetic studies it was decided to inject guinea pigs at 24 h before the challenge with nerve agent. The concentration of BuChE that circulated in blood at 24 h after administration of the enzyme depended on the dose and ranged between 200 and 800 nM. If it is assumed that the amount of blood is 6% of the body weight, the amount of BuChE circulating in blood as percentage of the administered amount can be calculated. These data are shown in the last column of Table 43. The values that we derived in our pharmacokinetic experiments are much lower than those calculated from the results obtained by other researchers (see TO 1 and 10). Only 9-16 % of the administered BuChE was recovered in blood.

Table 43 Maximum concentration ± SEM and recoveries of HuBuChE in blood of various animals after i.m. injection of HuBuChE

	Animal	Dose (nmol/kg)	Tmax (h)	Cmax (nmol/mL)	% HuBuChE in blood at Tmax
Raveh *et al*, 1993	mouse	160	10	0.43	16
Raveh *et al*, 1993	rat	160	10	0.83	31
Raveh *et al*, 1993	monkey	38	10	0.28	44
Allon *et al*, 1998	guinea pig	95	24	0.56	35
Ashani 1993	guinea pig	33*	24	16.7*	50
Ashani 1993	mouse	4.3*	12	1.25*	28
Ashani 1993	mouse	16.7*	12	3.33*	20
Ashani 1993	rat	36.7*	12	16.7*	45
Ashani 1993	rhesus monkey	133*	12	93*	70
Lenz 2004	guinea pig	234	26	0.19 ?	5 ?
Lenz 2004	rhesus monkey	64	10	0.37	33
TO 1	guinea pig	345	24	0.56	9
TO 2	guinea pig	200	24	0.68 ± 0.08	20 ± 2
TO 3	guinea pig	200	24	0.88 ± 0.24	26 ±7
TO 4	guinea pig	200	24	0.73 ± 0.24	21 ± 7
TO 5	guinea pig	200	24	0.51 ± 0.16	18 ± 4
TO 6	guinea pig	100	24	0.46 ± 0.07	27 ±5
TO 7	guinea pig	100	24	0.44 ± 0.05	26 ±3
TO 8	guinea pig	100	24	0.35 ± 0.05	20 ±2
TO 9	guinea pig	100	24	0.27 ± 0.10	16 ±5
TO10	marmoset	87- 116	12-16	0.31 ± 0.03	16 - 21
TO13	marmoset	71	16	0.15 ±0.06	13 ± 3
TO18	guinea pig	various	24		30 ± 2

* Amount of enzyme is given as nmol per animal

At the beginning of the toxicokinetic experiments, the concentration BuChE was also measured. From these measurements we found that approximately 22% was recovered in blood. This

number is still much lower than the recoveries found by other researchers. It must be remarked that the amounts that we injected i.m. were at least two times higher than the doses used by other researchers. The amount of enzyme that was administered was checked by the Ellman assay and the BuChE concentration in blood was measured according to the same protocol. Standard addition experiments of known amounts of enzyme to the blood matrix revealed that the blood matrix did not interfere with the results of the Ellman assay.
In another series of experiments guinea pigs were i.m. injected with HuBuChE, after 24 h they were euthanized, after which the BuChE activity was measured in the organs. The results are summarized in Table 35. In these experiments 30 % of the administered dose was recovered in blood and another 3 % was estimated to remain in the extravascular compartments of lungs, heart, brain and kidney. This means that the fate of 67% of the administered BuChE after 24 h is still unknown. It is possible that the enzyme is eliminated. However that would mean that the half life of BuChE is lower than 24 h which is not in agreement with the pharmacokinetic data.
Other research groups performed intravenous pharmacokinetics of HuBuChE but did not measure the BuChE activity in the organs. Raveh *et al* (1993) presented pharmacokinetic parameters pertaining to i.v. and i.m. administered HuBuChE and concluded that the fractions, absorbed following i.m. injections in rats was 0.9, based on the ratio of the AUC of i.m. and i.v. administration. This high ratio was found by calculation of the AUC that ranged from 0-70 hr. If the ratio of the AUCs between 0 and 10 h was calculated, the result would be much lower (estimated 0.40 for rats). In fact the latter value is more representative for the bioavailability of the scavenger at the time of the nerve agent challenge.
The pharmacokinetics of HuBuChE after i.m. administration in marmosets evolved significantly faster. The maximum HuBuChE concentration in blood was reached after 12 h and in one animal after 16 h. The elimination of HuBuChE proceeded also rather slow characterized by a half life of 53 h. The amount of BuChE that was recovered in blood ranged between 16 and 21%.

IV.2 Effect of HuBuChE pretreatment of guinea pigs on the toxicokinetics of soman

IV.2.1 Intravenous toxicokinetics of soman

Within the context of cooperative agreements DAMD17-85-G-5004, DAMD17-87-G-7015 and DAMD17-90-Z-0034 we studied the intravenous toxicokinetics of soman in anesthetized, atropinized and mechanically ventilated guinea pig at doses corresponding with 6 LD50, 2 LD50 and 0.8 LD50. Table 44 shows a summary of the toxicokinetic data of the intravenous toxicokinetic studies of soman that were performed in the past and during this study.
The concentrations of soman in blood that were measured after i.v. injection of soman corresponding with 2 LD50 were much lower than the concentrations found in earlier work. The ratio of the concentration-time courses of the C(+)P(-)-isomer and the C(-)P(-)-isomer are also larger than in the previous study. This difference is clear in Figure 3 and the values of the AUCs shown in Table 44. Such a higher stereoselectivity is typical for lower doses of soman. With lower doses the stereospecific binding to CaE becomes more apparent, which results in a higher stereoselectivity. With higher doses of soman, the CaE sites are more rapidly saturated which means that the remaining soman must be eliminated by enzymatic hydrolysis, which is not stereoselective with regard to the C(+)P(-) and C(-)P(-) isomers. A feasible reason might be that the dose of soman was lower than intended. However the standard solution of soman was checked and no diluting error could be found, which means that these animals were injected with the intended dose of 55 µg/kg. The toxicokinetics mentioned in DAMD17-87-G-7015 have been measured in 1989, which was more than 14 years ago at the time that we measured the toxicokinetics of soman in this study again (2003). Since guinea pigs are outbred animals, it is possible that the constitution of the specific enzymes has changed during the years. Therefore it

is better to compare the toxicokinetic data of the treated and non-treated animals. Figure 30 shows the concentration-time curves of C(-)P(-)-soman after i.v. injections of soman in naive and HuBuChE-pretreated animals.

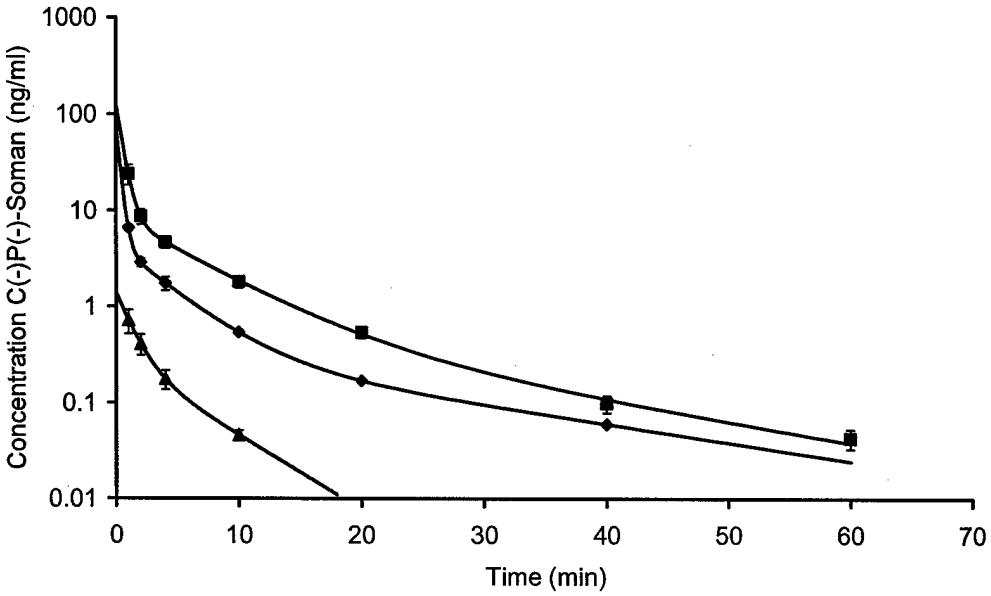

Figure 30 Blood levels of C(-)P(-)-soman after i.v-administration of C(±)P(±)-soman to anesthetized, atropinized, and artificially ventilated guinea pigs that had been pretreated (i.m.) 24 h earlier with human butyrylcholinesterase at a dose corresponding on a molar scale with 70% of a 2LD50 dose (i.v.) of C(±)P(±)-soman. Curve A (▲; AUC = 2.9 ± 0.6 ng.min.mL^{-1}; n=4, ±SEM) pertains to pretreated animals that were challenged (i.v.) with a single dose of C(±)P(±)-soman corresponding with 2 LD50 (55 µg/kg). Curve B (♦; AUC = 46 ± 1 ng.min.mL^{-1}; n= 2, ± SEM) pertains to non-pretreated animals challenged (i.v.) with the same dose of C(±)P(±)-soman. Curve C (■; AUC = 122 ± 26 ng.min.mL^{-1}; n=5, ± SEM) pertains to animals that were challenged with a second dose of 2 LD50 of C(±)P(±)-soman at 90 min after the first challenge.

It is clear that the concentrations of C(-)P(-)-soman in HuBuChE-pretreated animals are much lower than in naive animals. The effect of the scavenger is also clearly reflected in the values of the AUC. The difference in AUC of the P(-)-isomers between naive and HuBuChE-pretreated animals is 65.9 – 2.9 = 63 ng.min.mL^{-1} which is equal to 0.34 nmol.min.mL^{-1}. The time that acutely toxic levels of soman exist is reduced from 40 to 1 min. An explanation for the calculation of this acutely toxic level is shown in Appendix 2. The effect of the scavenger is also visualized in the higher clearance (Cl) and volume of the central compartment (Vl). The latter parameter is an indication that soman was extremely rapidly eliminated, which is expected for this scavenger.

Table 44 The effect of pretreatment with HuBuChE on the toxicokinetic parameters for soman in anesthetized, atropinized and artificially ventilated guinea pigs after i.v. administration

Parameter	Dimension	6 LD50 i.v. (a)	6 LD50 i.v. (a)	2 LD50 i.v. (b)	2 LD50 i.v.(b)	0.8LD50 i.v. (c)	0.8LD50 i.v.(c)	2 LD50 i.v. (d)	2 LD50 i.v. (d)	2 LD50 i.v. (d)	2 LD50 i.v. (d)	2 x 2LD50 (d)	2 x 2LD50 (d)
Dose	µg/kg	45.3	37.2	15.1	12.4	6.04	4.96	15.1	12.4	15.1	12.4	30.2	24.8
HuBuChE	nmol/kg	-	-	-	-	-	-			200	200	200	200
		C(+)P(-)	C(-)P(-)	C(+)P(-)	C(-)P(-)	C(+)P(-)	C(-)P(-)	C(+)P(-)	C(-)P(-)	C(+)P(-)	C(-)P(-)	C(+)P(-)	C(-)P(-)
Number of exponents		3	3	3	3		2	2	3		2	2	3
A	$ng.mL^{-1}$	339	317	318	354		3.8	2.05	70.0	-	1.11	20.0	119
B	$ng.mL^{-1}$	35	35	11	15		0.15	0.658	4.05	-	0.274	0.319	8.14
C	$ng.mL^{-1}$	2.79	9.57	1.0	1.7			-	0.356		-	-	0.75
a	min^{-1}	3.8	3.5	3.8	3.9		0.95	0.277	3.11	-	0.815	0.455	1.96
b	min^{-1}	0.12	0.16	0.19	0.21		0.12	0.0536	0.256	-	0.179	0.0316	0.176
c	min^{-1}	0.034	0.046	0.033	0.042		-	-	0.0445		-	-	0.0498
AUC	$ng.min.mL^{-1}$	458	516	172	203	2.0	5.25	19.6	46.3	-	2.89	54.1	122
C0	$ng.mL^{-1}$	377	362	329	369		3.95	2.29	74.4	0	1.38	20.3	128
t½,a	min	0.182	0.198	0.182	0.177		0.729	1.2	0.22	-	0.85	1.52	0.35
t½,b	min	5.77	4.33	3.65	3.30		5.77	14.7	2.7	-	3.87	21.9	3.94
t½,c	min	20.3	15.0	21.0	16.5		-	-	15.5				13.9
Vl	$l.kg^{-1}$	0.120	0.102	0.0458	0.0336		1.255	6.59	0.16		8.98	1.48	0.193
Cl	$l.min.kg^{-1}$	0.098	0.072	0.087	0.0611		0.944	0.767	0.267	-	4.28	0.558	0.203
Acutely toxic level until	min		140		100		2		40		1		50

(a) Data from DAMD 17-85-G-5004, (b) Data from DAMD17-87-G-7015, (c) Data from DAMD17-90-Z-0034, (d) Data from this report, (e) After administration of C(±)P(±)-soman. It is assumed that the area under the curve of 1.3 $ng.mL.min^{-1}$ for C(±)P(-)-soman is the minimum area with toxicological relevance. See Appendix 2 for explanation.

After one injection of soman only the C(-)P(-) isomer of soman could be detected in the HuBuChE-pretreated guinea pigs. The C(+)P(-)- isomer had completely disappeared from the blood stream. This result was expected in view of the higher binding constant of C(+)P(-) with BuChE compared to the binding constant of the C(-)P(-) isomer with BuChE (*vide infra*).
In another experiment the first injection of soman was followed by a second injection of soman at 90 min after the first injection. Figure 30 shows that the concentration of C(-)P(-)-soman was higher than that in non-pretreated animals. Apparently after the first dose of soman, less binding sites are available than in guinea pigs to which no HuBuChE or soman was administered. The AUC of the guinea pig that received two doses of soman is still lower than the AUC of the guinea pig from the toxicokinetic study that was performed in 1989 (DAMD17-87-G-7014). Apparently there were still binding sites available. Figure 6 shows that the blood samples taken at 90 min after the first injection contained a significant amount of BuChE. After the second injection of soman the concentration of BuChE in blood was negligible.

IV.2.2 Inhalation toxicokinetics of (±)-soman

Guinea pigs

The toxicokinetics of inhaled soman have been measured previously within the framework of DAMD17-90-Z-0034. During that study guinea pigs were exposed to sub-lethal doses of soman vapor in air. The exposure time varied between 4 and 8 min and the concentration was adjusted to reach a Ct of 0.8 LCt50. During the present study the exposure time was shortened to 2 min, because this exposure time was considered as a more realistic scenario, *i.e.* a short exposure time and a relatively high vapor concentration. Initially it was intended to compare the results of the toxicokinetics of soman in HuBuChE-pretreated guinea pigs that were exposed to 2 LCt50 with the results of the toxicokinetics of soman in naive guinea pigs that were exposed with an equivalent of 0.8 LCt50. During the grant period it was realized that it was better to study the effect of the scavenger in naive and HuBuChE-pretreated animals, because the toxicokinetics measured many years ago were not a good reference. Secondly, when the effect of the scavenger is to be studied, it is better to change only one parameter of the experiments at a time, *i.e.* with or without pretreatment with HuBuChE and not also the exposure conditions. It was intended to pretreat animals with HuBuChE and expose them to soman with a Ct corresponding with 2 LCt50. As mentioned in section III.5, that would require a vapor concentration of 480 $mg.m^{-3}$ for a 2-min exposure. It was not likely that the animals would survive this exposure if not pretreated with HuBuChE. Because the goal of this project is to show the effect of the scavenger on the toxicokinetics and not the efficacy on survival rates, it was decided to lower the vapor concentration in order to be sure that the animals would survive the exposure regardless of scavenger pretreatment. It was chosen to expose the animals for 2 min to a soman vapor concentration of 200 $mg.m^{-3}$.
It was not possible to construct averaged exponential toxicokinetic curves because the deviations of the soman concentrations in blood were too high. Therefore the curves were separately evaluated and the AUCs were calculated using the trapezium method. Table 45 shows a summary of the exposure conditions and the AUCs.

Table 45 Exposure conditions and AUC of C(+)P(-)- and C(-)P(-)-soman for toxicokinetic experiments in which naive and HuBuChE-pretreated guinea pigs were nose-only exposed to soman vapor in air

Toxicity equivalent	Vapor conc ($mg.m^{-3}$)	Exposure time (min)	Ct ($mg.min.m^{-3}$)	HuBuChE (nmol/kg)	Isomer	AUC ($ng.min.mL^{-1}$)
0.8 LCt50*	96	4	384	0	C(+)P(-)	4.3
0.8 LCt50*	96	4	384	0	C(-)P(-)	7.7
0.8 LCt50*	48	8	384	0	C(+)P(-)	16
0.8 LCt50*	48	8	384	0	C(-)P(-)	24
	200	2	400	0	C(+)P(-)	5.6-843
	200	2	400	0	C(-)P(-)	17-165
	200	2	400	100	C(+)P(-)	0
	200	2	400	100	C(-)P(-)	0.3-6
	200	2	400	100	C(+)P(-)	0
	200	2	400	100	C(-)P(-)	0
	2	300	600	100	C(+)P(-)	0
	2	300	600	100	C(-)P(-)	0

*Data from DAMD17-90-Z-0034

Only two animals that were not pretreated with HuBuChE were exposed for 2 min to soman vapor. One animal showed very high levels of soman in which the concentrations of C(-)P(-)-soman were higher than those of the C(+)P(-)-soman isomers. However the data points for 20 and 40 min showed higher concentrations of C(+)P(-)-soman than for the C(-)P(-)- isomer. This behavior was not expected and was not reproducible. Guinea pig 2 showed much lower concentrations of soman in blood and the AUC of the C(-)P(-)- and C(+)P(-) isomers were 17 and 5.6 $ng.min.mL^{-1}$ respectively. These values are higher than those obtained after a 4-min exposure but lower than obtained after an 8-min exposure. In HuBuChE-pretreated animals the C(+)P(-)-soman isomer was not detected. This was expected for the same reasons as discussed in IV.2.1. The AUC values of the C(-)P(-)-soman isomer ranged from 0.3 to 6 $ng.min.mL^{-1}$. This value is significantly lower than the values for the non-pretreated animals. Table 16 shows that all animals had a residual concentration of active BuChE in blood. Presumably the amount of inhaled soman was not high enough to occupy all binding sites. The 2-min exposure might be a realistic scenario but it appeared to be not very reproducible because of variation in the respiratory volume of the animal. Unfortunately the respiratory minute volume was not measured. The variation in inhaled soman was also verified by the amount of soman that could be regenerated upon incubation with fluoride ions. A low amount of regenerated soman indicated a low amount of inhaled soman and a high concentration of residual BuChE.
The toxicokinetics of intact soman during a 300-min exposure of HuBuChE-pretreated guinea pigs to a low level concentration of soman vapor could not be measured. If soman was detected in a particular blood sample, the animal was dead at the time point that the next blood sample was to be taken. Consequently, the toxicokinetics existed of only one data point. More information was gathered from the results of the fluoride reactivation assay as will be discussed in section IV.5.

Marmosets

In the past, the toxicokinetics of soman in marmosets have only been studied after intravenous administration. According to the protocol the effect of HuBuChE as a scavenger on the toxicokinetics of soman was to be studied in marmosets that were exposed for 2 min to soman vapor in air. It was proposed to expose non-pretreated marmosets to 0.8 LCt50 and pretreated animals to a Ct corresponding with 2 LCt50. The LCt50 has not been determined and was estimated to be 2.7 times lower than the LCt50 for guinea pigs, based on the ratio of the i.v. LD50 of the two animals. For a 2-min exposure, a vapor concentration of 68 $mg.m^{-3}$ would be required for 0.8 LCt50 and 171 $mg.m^{-3}$ would be required for 2 LCt50. During the study it was decided that it would be better to study the effect of the scavenger in animals that were exposed under the same conditions. At the same time it was required that the animals would survive the nerve agent challenge, with or without pretreatment. The results are shown in section III.7. Eventually it was decided to challenge the animals for 5 min to 50 $mg.m^{-3}$ soman vapor. The deviation between the curves was quite high. Therefore, the AUCs were calculated for each experiment, mostly by integration according to the trapezium method. Table 46 shows a summary of exposure conditions and the AUCs.

Table 46 Exposure conditions and AUC of C(+)P(-)- and C(-)P(-)- soman for toxicokinetic experiments in which naive and HuBuChE-pretreated marmosets were nose-only exposed to C(±)P(±)-soman vapor in air

Toxicity equivalent	Vapor conc ($mg.m^{-3}$)	Exposure time (min)	Ct ($mg.min.m^{-3}$)	HuBuChE (nmol/kg)	isomer	AUC ($ng.min.mL^{-1}$)
2 LD50*			20 µg/kg (i.v.)	0	C(+)P(-)	81
2 LD50*			20 µg/kg (i.v.)	0	C(-)P(-)	85
	50	2	100	0	C(+)P(-)	0-14.9
	50	2	100	0	C(-)P(-)	0-3.6
	50	5	250	0	C(+)P(-)	4.6-30.6
	50	5	250	0	C(-)P(-)	2.1-23.6
	50	5	250	100	C(+)P(-)	0-2.4
	50	5	250	100	C(-)P(-)	0-3.6

*Data from DAMD17-87-G-7015

It is remarkable that the AUC of the C(+)P(-) isomers was higher than for the C(-)P(-)-isomers in the non-pretreated animals. This phenomenon was observed in three non-pretreated animals that were exposed to soman. The maximum AUC that was observed upon exposure for 5 min to 50 $mg.m^{-3}$ was 37% of the AUC pertaining to a 2 LD50 exposure. The AUC values of the HuBuChE-pretreated animals are all much lower than for the naive animals. In this case the concentration of the C(+)P(-) isomers are lower than for the C(-)P(-)- isomers, which is expected in view of the higher binding constants of the C(+)P(-) isomer with HuBuChE. The decrease in AUC for the C(±)P(-) isomers was 54.2 – 6.0 = 48.2 $ng.min.mL^{-1}$ which is equal to 0.26 $nmol.min.mL^{-1}$.

IV. 3 Effect of HuBuChE pretreatment on toxicokinetics of (±)-sarin

IV.3.1 Intravenous toxicokinetics of sarin

Within the context of cooperative agreement DAMD17-90-Z-0034 we have studied the intravenous toxicokinetics of (±)-sarin in the anesthetized, atropinized and mechanically ventilated guinea pig at a dose corresponding with 0.8 LD50 (19.2 µg/kg) (Benschop and Van Helden, 1993). The intravenous toxicokinetics were mainly studied as an 'equitoxic' reference for

the inhalation toxicokinetics at 0.8 LCt50 in an 8-min exposure. When we conceived the proposal for the current study on the effect of pretreatment with HuBuChE on the toxicokinetics of nerve agents we intended to use the previously measured toxicokinetic curve for 0.8 LD50 (±)-sarin in the naive guinea pig as a reference for the curve of 2 LD50 (±)-sarin in the HuBuChE-pretreated guinea pig, assuming a dramatic effect of HuBuChE-pretreatment on the toxicokinetics.

When performing the experiments, the effect on the toxicokinetics appeared not to be that dramatic. As a result, we decided to perform a limited study on the intravenous toxicokinetics of (±)-sarin for a dose corresponding with 2 LD50 in naive guinea pigs to provide an adequate reference for the experiments in HuBuChE-pretreated animals.

As was the case for an intravenous dose corresponding with 0.8 LD50, the (+)-isomer of sarin was not detectable in the blood of the guinea pigs after an i.v. bolus of a dose corresponding with 2 LD50. The half-life of distribution appears to be 0.15 min after a dose of 0.8 LD50 and 0.98 min after 2 LD50, whereas the terminal half-lives resulting from these doses are 58 and 389 min, respectively. The calculated AUC-values are 15.3 ng.min.mL^{-1} for 0.8 LD50 and 109 ng.min.mL^{-1} for 2 LD50, indicating non-linearity of the toxicokinetics with the dose, as was also observed for 0.8 and 2 LD50 of C(±)P(±)-soman (Benschop and Van Helden, 1993).

Previously, we already concluded that (-)-sarin is more persistent in the guinea pig than C(±)P(-)-soman at an i.v. dose corresponding with 0.8 LD50 (Benschop and Van Helden, 1993). This is also true for an i.v. dose of (±)-sarin corresponding with 2 LD50.

Table 47 The effect of pretreatment with HuBuChE on the toxicokinetic parameters for (-)-sarin in anesthetized, atropinized and artificially ventilated guinea pigs after i.v. administration of (±)-sarin

Parameter	Dimension	0.8LD50 (a) i.v.	2 LD50 i.v.	2 LD50 i.v.	2LD50 i.v.
Dose (µg/kg)	µg/kg	9.6	24	24	24
HuBuChE (nmol/kg)	nmol/kg	-	-	200	200
		P(-)	P(-)	P(-)	P(-)
Number of exponents					
A	ng.mL^{-1}	35.9	17.1	4.80	
B	ng.mL^{-1}	0.09	0.151	0.116	
a	min^{-1}	4.6	0.705	0.42	
b	min^{-1}	0.012	0.00178	0.00637	
AUC	ng.min.mL^{-1}	15.3	109	29	
AUC (1'-60')	ng.min.mL^{-1}	3.83	20.4	13.0	2.3
C0	ng.mL^{-1}	36.0	17.3	5.22	
t½,a	min	0.15	0.98	1.65	
t½,b	min	57.8	389	108	
Vl	l.kg^{-1}	0.26	1.38	4.59	
Cl	l.min.kg^{-1}	0.62	0.22	2.14	
Acute toxic level until *	min	10	480	140	< 1

* After administration of (±)-sarin. It is assumed that the area under the curve of 6.9 ng.mL.min^{-1} for (-)-sarin is the minimum area with toxicological relevance. See Appendix 2 for explanation.

(a) Data from DAMD17-90-Z-0034

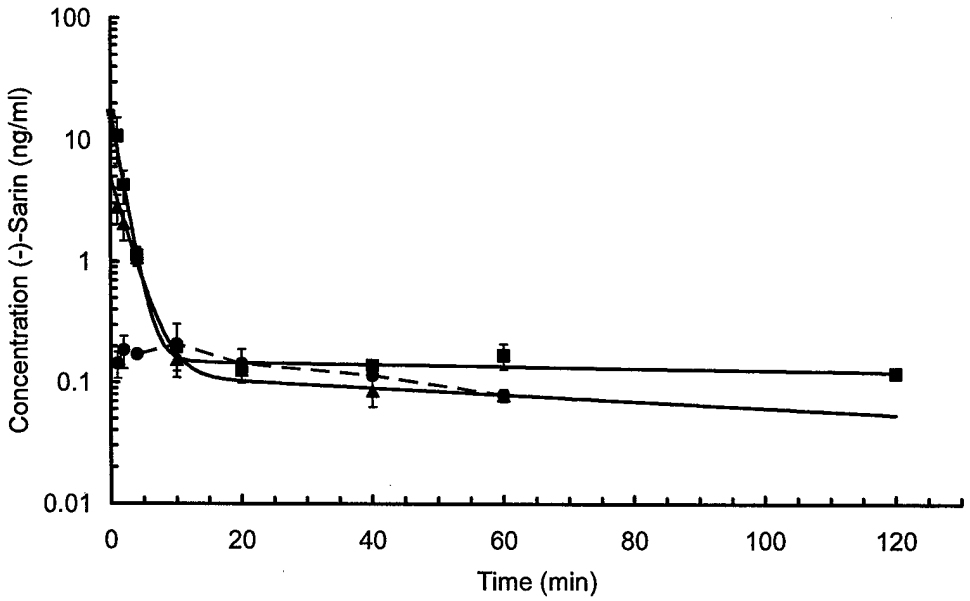

Figure 31 Mean concentration-time course of (-)-sarin (ng/mL ± SEM, n=4) in blood of anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. administration of a (±)-sarin dose of 48 µg/kg, which corresponds with 2 LD50. Naive animals (■), HuBuChE-pretreated animals 1, 4 (▲), HuBuChE-pretreated animals 2, 3, 5 (●).

In Figure 31 the measured toxicokinetic curves in naive guinea pigs and HuBuChE-pretreated animals are shown. The curves of the non-pretreated animals were averaged. The curves of the HuBuChE-pretreated animals were divided into two groups. The average of the blood levels of guinea pigs 1 and 4 show almost the same curve as the non-pretreated animals. However, the data in Table 47 show that there is a significant difference in AUC between the two curves (109 and 29 ng.min.mL^{-1}). The effect of the scavenger is also visualized by the increase of the fictive volume of the central compartment and more rapid clearance. It must be mentioned that a major part of the AUC is still contributed by the terminal phase of the toxicokinetic curve. The AUC(1-60') is 20.4 and 13.0 for the naive and HuBuChE-pretreated animals respectively. In the other group of HuBuChE-pretreated animals (confer Fig.31) the concentration of sarin was already very low in the first sample that was taken after injection of sarin. It must be mentioned that guinea pigs 1 and 4 received a slightly lower dose of HuBuChE than guinea pigs 2, 3 and 5 (160 nmol/kg for guinea pigs 1,4 and 196 ±27 nmol/kg for guinea pigs 2, 3 and 5). The residual activities of BuChE in guinea pigs 1 and 4 were quite low, while the residual concentration of BuChE in blood of guinea pigs 2, 3, and 5 was quite high, which is plausible because there was no sarin present to inhibit BuChE.

It is remarkable that the concentrations of (-)-sarin remain rather constant at a relatively low level for a long period of time. We cannot explain at this point why these concentrations are not reduced faster by the still relatively high BuChE concentrations circulating in blood. The time that acute toxic levels of sarin circulate is very high because of the high persistence of sarin. Nevertheless the time that acute levels of sarin still circulate is reduced from 480 to 140 min and in some cases even to less than 1 min upon pretreatment with HuBuChE. It must also be mentioned that the argumentation is based on the assumption that completely inhibited AChE is

reactivated by oximes (see Appendix 2). In this case it is presumable that the activity of AChE is higher and in equilibrium with inhibited AChE.

IV.3.2 Inhalation toxicokinetics of (±)-sarin

In view of the rapid death of two out of three animals, Haber's rule appeared to be not applicable for converting the 24-h LCt50 of (±)-sarin for an 8-min nose-only exposure to a value for a 2-min exposure. More likely the 'toxic load principle' (C^n*t=constant) should be used for this conversion. The value of 'n' is generally accepted to be *ca.* 1.5, as derived from a study by Cresthull *et al.* (1957) in which monkeys were exposed to G-agent vapors in 2 and 10 min. In order not to waste more animals we decided more or less intuitively to lower the concentration of (±)-sarin vapor from 375 $mg.m^{-3}$ to 200 $mg.m^{-3}$, which corresponds with a value of 'n' of 1.8. Applying n=1.5 would suggest exposure to 237 $mg.m^{-3}$ for 2 min. Consequently, exposure to 200 $mg.m^{-3}$ for 2 min may therefore be equivalent to somewhat less than 2 LCt50. Since the toxicokinetics are studied in both naive and HuBuChE-pretreated guinea pigs under these conditions, this deviation is not a major problem.

The dose of HuBuChE to be used for pretreatment in the inhalation toxicokinetic studies was calculated using values for the respiratory minute volume and the retention of C(±)P(±)-soman in anesthetized and atropinized guinea pigs that we actually measured (Trap, 2002). Allon *et al.* (1998) have used a different approach to calculate the desired dose of HuBuChE. In their experiments the guinea pigs were fully protected against *ca.* 2.5 inhaled LD50s of C(±)P(±)-soman at a dose of HuBuChE that is rather close to the one we use. This means that the dose we calculated is in the right ballpark.

An interesting finding in the toxicokinetic study is that we were able to quantify (+)-sarin in blood after the 2-min exposure to concentrations of (±)-sarin vapor in air of 376 and 200 $mg.m^{-3}$, which was not the case during and after 8-min exposure to 19 and 38 $mg.m^{-3}$. (Benschop and Van Helden, 1993). Apparently the rate of absorption from the (upper) airways is higher than the rate of detoxification under these conditions. As anticipated, the (+)-isomer subsequently disappears more rapidly from the blood than the (-)-isomer, most likely due to hydrolysis. In inhalation experiments with C(±)P(±)-soman we did measure traces of one of the P(+)-isomers during 8-min exposure to 48 $mg.m^{-3}$ of C(±)P(±)-soman vapor in air (corresponding with 0.8 LCt50) but oddly enough not during or after a 4-min exposure to a twofold higher concentration (Benschop and Van Helden, 1993).

Another interesting finding is the increase in (-)-sarin concentration in the first few minutes after ending the exposure to the nerve agent vapor, which we observed in the majority of the animals (HuBuChE-pretreated as well as naive). This suggests that the absorption of the agent continues after ending the exposure, probably from a deposition site in the upper airways. This phenomenon was not observed for 8-min exposure to 0.4 and 0.8 LCt50 of (±)-sarin, neither for 8-min exposure to 0.4 and 0.8 LCt50, nor for a 4-min exposure to 0.8 LCt50 of C(±)P(±)-soman (Benschop and Van Helden, 1993). We did however observe that the concentrations of the P(-)-soman isomers remained higher after ending the nose-only exposure than after ending an equitoxic intravenous infusion, which also implies continuation of absorption after ending the exposure.

In nearly all of the intravenous and inhalation toxicokinetic curves, the concentration of (-)-sarin does not continue to decrease in the terminal phase of the toxicokinetics but in many cases appears to level off or even increase slightly at 1-2 h after administration or exposure. A similar phenomenon was observed for the toxicokinetics of 0.8 LD50 (i.v.) and 0.4 LCt50 (8-min nose-only exposure) of (±)-sarin (Benschop and Van Helden, 1993). At that time we considered the increasing (-)-sarin concentration near the end of the toxicokinetic curve to be an analytical artifact, resulting from the fact that the concentrations were approaching the detection limit of

the analytical procedure. However, in view of the results obtained in the current study, this phenomenon can no longer be ignored. Interestingly, we hardly ever have seen the concentrations of either of the P(-)-isomers of soman to increase in the terminal phase. As most of the toxicokinetic experiments within the context of the current study are devoted to C(±)P(±)-soman, it will become apparent whether this phenomenon is actually associated with (±)-sarin and not with C(±)P(±)-soman. Tentative explanations for the leveling-off or increase of the (-)-sarin concentration in the terminal phase of the toxicokinetics are release from rather a specific, non-covalent binding sites, or reactivation from covalent binding sites induced by endogenous fluoride ions. The apparent persistence of (-)-sarin may have important implications for the approaches to pretreatment and therapy of intoxications by this nerve agent.
In Figure 32 all ten curves measured for nose-only exposure to 200 $mg.m^{-3}$ of (±)-sarin vapor in air are presented. Although this is a very crowded graph, it is clear that there is no dramatic difference between the concentration-time courses of (-)-sarin in naive and HuBuChE-pretreated animals. In fact, the lower curves measured in naive animals overlap with the higher curves measured in HuBuChE-pretreated animals.

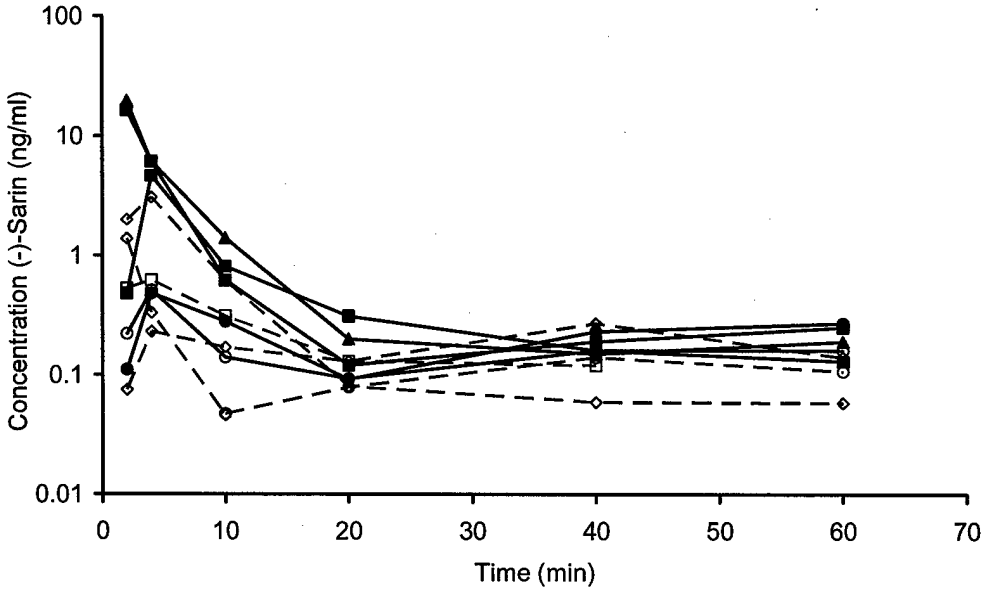

Figure 32 Concentration-time courses of (-)-sarin (ng/mL) in anesthetized and atropinized guinea pigs after a 2-min nose-only exposure to 200 $mg.m^{-3}$ (±)-sarin vapor in air. HuBuChE-pretreated animals: open markers, dotted lines; non-pretreated animals: filled markers, solid lines

In the terminal phase the concentrations of (-)-sarin measured in the two groups of guinea pigs are very close together. The situation is summarized in Table 48 which shows the AUC of the various exposures of pretreated and non-pretreated animals. The variation of values of the AUC of the non-pretreated animals that were exposed for 2 min is rather large, which is again an indication that a short exposure to a high concentration of sarin vapor might be realistic but is not very reproducible. Nevertheless the AUCs of the toxicokinetic experiments in which HuBuChE-pretreated animals were exposed were lower than those of the naive animals, however there is an overlap between the two groups.

Table 48 Exposure conditions and AUC of (-)-sarin for toxicokinetic experiments in which naive and HuBuChE-pretreated guinea pigs were nose-only exposed to sarin vapor in air

Toxicity equivalent	Vapor Conc ($mg.m^{-3}$)	Exposure time (min)	Ct ($mg.min.m^{-3}$)	HuBuChE (nmol/kg)	AUC (0-120') ($ng.min.mL^{-1}$)	AUC ($0\rightarrow\infty$) ($ng.min.mL^{-1}$)
0.8 LCt50*	38	8	304	0	9.1	15.4
0.4 LCt50*	19	8	152	0	5.5	58.5
	376	2	752	83	5.6- 161	
	200	2	400	0	7-61	
	200	2	400	84	4-22	

*Data from DAMD17-90-Z-0034

IV. 4 Effect of HuBuChE pretreatment on toxicokinetics of VX

IV.4.1 Intravenous toxicokinetics

The toxicokinetics of VX were already measured within the framework of DAMD17-97-2-7001. The effect of pretreatment with HuBuChE is shown in Figure 33, which shows the concentrations of VX in blood of guinea pigs that were not pretreated and of guinea pigs that were pretreated with 200 nmol/kg HuBuChE.

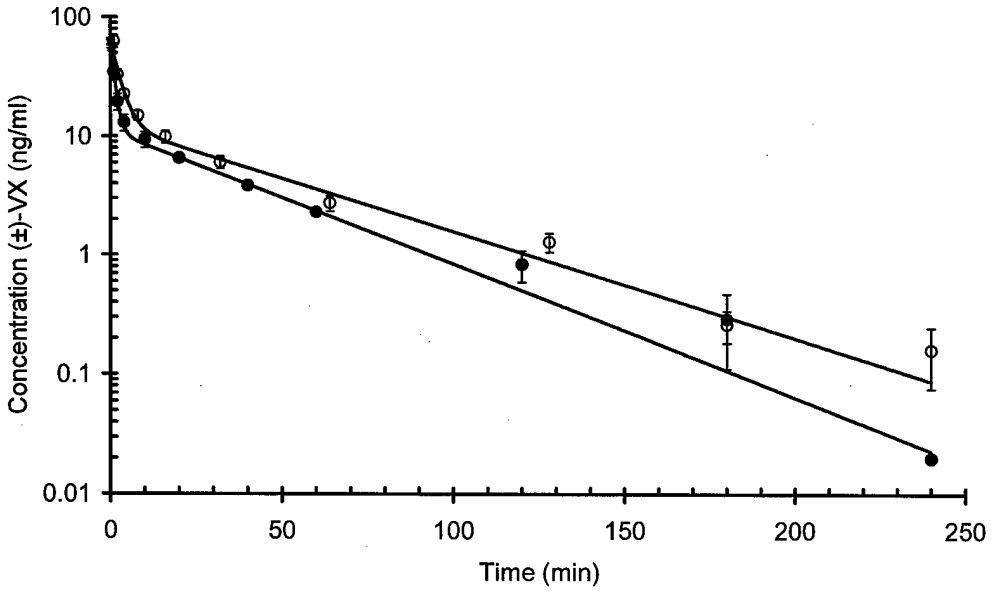

Figure 33 Concentration of VX (ng/mL) in blood of anesthetized, atropinized and artificially ventilated hairless guinea pigs after i.v. administration of VX (56 µg/kg) corresponding with 2LD50. Naive animals (○), HuBuChE-pretreated animals 200 nmol/kg (●).

The VX levels in blood of HuBuChE-pretreated guinea pig are significantly lower, but according to the curves this effect seems not to be very large. Table 49 shows a summary of the toxicokinetic parameters of this study and the toxicokinetic study performed in the previous grant.

Table 49 The effect of pretreatment with HuBuChE on the toxicokinetic parameters for (±)-VX in anesthetized, atropinized and artificially ventilated guinea pigs after i.v. administration of (±)-VX

Parameter	Dimension	1LD50 i.v.*	2LD50 i.v.*	2 LD50 i.v.	2 LD50 i.v.
Dose	µg/kg	28	56	56	56
HuBuChE	nmol/kg	-	-	200	130
Number of exponents		2	2	2	2
A	$ng.mL^{-1}$	13.8	51.0	54.2	58.45
B	$ng.mL^{-1}$	11.5	12.4	11.01	12.68
a	min^{-1}	0.417	0.35	0.817	0.5709
b	min^{-1}	0.038	0.020	0.0257	0.0242
AUC	$ng.min.mL^{-1}$	335	765	494	626
C0	$ng.mL^{-1}$	25.3	63.4	65.2	71.1
t½,a	min	1.66	1.98	0.848	1.21
t½,b	min	18.2	34.6	26.9	28.6
Vl	$l.kg^{-1}$	1.10	0.883	0.858	0.787
Cl	$l.min.kg^{-1}$	0.0845	0.0745	0.113	0.0894
Acutely toxic levels until (a)	min	145	310	230	250

* Data from DAMD17-97-2-7001 (a) After administration of (±)-VX. It is assumed that the area under the curve of 0.6 $ng.min.mL^{-1}$ (-)-VX is the minimum area with toxicological relevance. See Appendix 2 for explanation. The ratio of the VX isomers is expected to be 1:1, which means that the AUC of 1.2 $ng.min.mL^{-1}$ of (±)-VX was used as the minimum area with toxicological relevance.

The effect of the scavenger is best reflected in the value of the AUC. Table 49 shows three experiments in which guinea pigs were administered with VX (55 µg/kg) which corresponds with 2 LD50. The decline of the AUC upon pretreatment with 200 nmol/kg HuBuChE was 765-494 = 271 $ng.min.mL^{-1}$, which is equal to 1.0 $nmol.min.mL^{-1}$. By comparison, the decrease of the AUC of C(±)P(-)-soman in guinea pigs administered with 55 µg/kg C(±)P(±)-soman was 0.34 $nmol.min.mL^{-1}$. The value of the AUC in the VX toxicokinetics consists of the sum of both isomers. The P(+)-isomers of soman were not detected but it cannot be excluded that the P(+)-isomers consumed a part of the scavenger. However it will not be enough to explain the difference in decrease of AUC of VX and soman. The AUCs of the VX curves are calculated over a time span of 240 min, while the AUC of the soman curves are calculated over a time span of 60 min. Table 50 shows the AUCs of both experiments expressed in $nmol.min.mL^{-1}$ for different time spans. Table 50 shows that the decrease of AUC as result of HuBuChE pretreatment is larger for the VX experiment than for the soman experiment.
The elimination of soman is caused by enzymatic hydrolysis and binding to CaE. The elimination of VX is driven by slow enzymatic hydrolysis and slow binding to CaE. Additional fast binding places such as BuChE will be consumed rapidly. Addition of fast binding sites to soman will only increase the number of multiple fast elimination routes. It is therefore explicable that the scavenger will not be consumed completely, which means that the effect of the scavenger on the toxicokinetics of soman is lower in this case. Table 49 shows clearly that the time period in which acutely toxic levels of VX circulate is reduced from 310 to 230 min after administration of 200 nmol/kg HuBuChE, but in fact it is not more than a than a drop in the ocean. The effect of the scavenger is also visualized in a moderate increase of the clearance.

Table 50 AUC (nmol.min.mL^{-1}) of (±)-VX and C(±)P(-)-soman in blood of naive or HuBuChE-pretreated anesthetized, atropinized and mechanically ventilated guinea pigs. Guinea pigs were i.v. injected with VX (56 µg/kg) or C(±)P(±)-soman (55 µg/kg).

	AUC of (±)-VX (nmol.min.mL^{-1})			AUC of C(±)P(-)-Soman (nmol.min.mL^{-1})		
Time (min)	without HuBuChE	HuBuChE (200 nmol/kg)	Difference as result of HuBuChE treatment	without HuBuChE	HuBuChE (200 nmol/kg)	Difference as result of HuBuChE treatment
0	0	0	0	0	0	0
0-30'	1.59	1.11	0.48	0.337	0.014	0.32
0-60'	2.16	1.51	0.65	0.356	0.015	0.34
0-90'	2.48	1.69	0.79	0.360	0.015	0.35
0-120'	2.65	1.78	0.87			
0-180'	2.80	1.84	0.96			
0-240'	2.84	1.85	0.99			

IV.4.2 Percutaneous toxicokinetics

In another series of experiments hairless guinea pigs were pretreated with HuBuChE and percutaneously exposed to a dose VX corresponding with 2LD50. Intact VX was not detected. The percutaneous toxicokinetics of VX in naive animals were measured within the framework of DAMD17-97-2-7001 but with a dose of VX corresponding with 1 LD50. These data are the only reference to compare the results of the HuBuChE-treated animals. In the previous section it was shown that 200 nmol/kg HuBuChE resulted in the reduction of the AUC of 271 ng.min.mL^{-1}. The AUC after application of VX corresponding with 1 LD50 in naive animals was 43 ng.min.mL^{-1}. Assuming linear toxicokinetics it is plausible that VX cannot be detected even after application of a higher dose VX. Moreover, the blood samples taken at the end of the experiment contained considerable concentrations of free BuChE, which means that it is unlikely that VX will be present in the same sample.

Table 51 Exposure conditions and AUC of toxicokinetic experiments in which naive and HuBuChE-pretreated hairless guinea pigs were percutaneously exposed to VX

Toxicity	Dose (µg/kg)	HuBuChE (nmol/kg)	AUC (ng.min.mL^{-1})
1 LD50	125	0	43 ± 8.3
2 LD50	250	100	0

It cannot be concluded that a treatment of 100 nmol/kg HuBuChE is sufficient to protect against percutaneous intoxication with VX at doses corresponding with 1 or 2 LD50. It is necessary to study the percutaneous toxicokinetics of VX more thoroughly, because the effects of the percutaneous VX application manifest itself after 6 h, which was beyond the time span (6 h) of this experiment.

IV.5 Interpretation of the results of the fluoride reactivation assay

The blood samples taken at the final time point in each toxicokinetic experiment were used for the fluoride reactivation assay. The phosphyl moiety of the nerve agent can be released from the enzyme (BuChE, CaE or other binding sites) upon incubation with fluoride ions. The released organophosphofluoridate is extracted and measured with GC. Table 52 shows an overview of all toxicokinetic experiments and the results of the fluoride reactivation assay.

Table 52 Concentration of regenerated organophosphofluoridates (ng/mL plasma and nM) in the blood samples taken at the final time points of the toxicokinetic experiments.

Agent	Animal	Route	Dose or Ct	HuBuChE (nmol/kg)	F-react (ng/mL)	F-react (nM)	n
Soman	Guinea pig.	i.v.	55 $\mu g.kg^{-1}$	0	57 ± 0.4	313 ± 2.2	2
Soman	Guinea pig	i.v.	55 $\mu g.kg^{-1}$	200	62 ± 3	341 ± 16	5
Soman	Guinea pig	i.v.	110 $\mu g.kg^{-1}$	200	98 ± 18	538 ± 98	6
Soman	Guinea pig	inhalation	2'; 200 $mg.m^{-3}$	0	62 ± 10	340 ± 55	2
Soman	Guinea pig	inhalation	2'; 200 $mg.m^{-3}$	100	51 ± 8	279 ± 44	7
Soman	Guinea pig	inhalation	300'; 2 $mg.m^{-3}$	0	35	192	1
Soman	Guinea pig	inhalation	300'; 2 $mg.m^{-3}$	100	91± 21	500 ± 115	4
Soman	Marmoset	inhalation	2'; 50 $mg.m^{-3}$	0	3.5 ± 0.3	19 ± 1.7	2
Soman	Marmoset	inhalation	5'; 50 $mg.m^{-3}$	0	3.2 ± 0.1	18 ± 0.6	2
Soman	Marmoset	inhalation	5'; 50 $mg.m^{-3}$	100	2.8 ± 0.1	15 ± 0.6	3
Sarin	Guinea pig	i.v.	48 $\mu g.kg^{-1}$	0	53 ± 14	379 ± 100	4
Sarin	Guinea pig	i.v.	48 $\mu g.kg^{-1}$	200	379 ± 18	2707 ± 130	3
Sarin	Guinea pig	inhalation	2'; 200 $mg.m^{-3}$	0	56 ± 6	403 ± 43	5
Sarin	Guinea pig	inhalation	2'; 200 $mg.m^{-3}$	100	241 ± 13	1721 ± 93	3
Sarin	Guinea pig	inhalation	2'; 376 $mg.m^{-3}$	100	391± 76	2793 ± 542	3
VX	Guinea pig	i.v.	55 µg/kg	200	85 ± 9	685 ± 73	4
VX	Guinea pig	percutaneous	250 µg/kg	0	1	8	1
VX	Guinea pig	percutaneous	250 µg/kg	100	15 ± 2	121 ± 16	3

The data from the fluoride reactivation assay provide positive verification of the exposure to the nerve agent. Such additional data can be important in the case that the exposure cannot be fully controlled, e.g. in case of nose-only or percutaneous exposure. The fluoride reactivation assay provides information about the degree of saturation of binding sites by the nerve agent. Thirdly the fluoride reactivation assay verifies the efficacy of the scavenger in case that the nerve agent can be released from BuChE, *i.e.* in the case of sarin or VX administration. Soman cannot be regenerated from BuChE with fluoride ions because of rapid aging of the phosphyl moiety.

Guinea pig – i.v. toxicokinetics of soman

The concentration of soman that could be regenerated from plasma of guinea pigs that were i.v. injected with soman (55 µg/kg) was approximately 57 ng/mL. Soman was regenerated from regenerable binding sites, presumably CaE. After the guinea pigs have been pretreated with HuBuChE and i.v. injected with the same dose of soman, the concentration of regenerated soman does not increase, because soman cannot be regenerated from BuChE. The dose of regenerated soman increased to 98 ng/mL when two doses of soman (2 x 55 µg/kg) were administered. This is an indication that the binding sites were not fully occupied after one dose of soman and presumably also not after two doses of soman.

Guinea pig – inhalation toxicokinetics of soman

The concentration of regenerated soman after a 2-min nose only exposure to 200 $mg.m^{-3}$ soman vapor was 61 ng/mL. The concentration of regenerated soman in HuBuChE-pretreated animals that were exposed under the same conditions was slightly lower. The lower concentration is probably caused by a lower amount of soman that was inhaled, which appeared to be quite variable between the animals. The concentration of regenerated soman in a blood sample taken after a 120 min exposure to 2 $mg.m^{-3}$ of soman vapor in naive animals was 35 ng/mL. The

animal was dead when the sample for time point 180 min was due to be taken. Presumably the concentration of regenerated soman would have been higher if the sample of time point 180 min was also analyzed.
The concentration of regenerated soman in HuBuChE-pretreated guinea pigs that were exposed to the same soman vapor concentration of 2 $mg.m^{-3}$ was 91 ng/mL, which is close to the concentration of regenerated soman that was found in plasma of animals that received 2 x 55 µg/kg i.v. Presumable the animals stayed alive for a longer exposure time because they were pretreated with HuBuChE, which means that they had more rapid binding sites available than naive animals. In this way, the essential AChE was saved and extended the life of the animal. This caused the animals to inhale a higher dose of soman, as reflected in the higher concentration of fluoride regenerated soman.

Marmoset – inhalation toxicokinetics of soman
The concentration of regenerated soman in plasma of the marmoset that was exposed to soman vapor was 3.5 ng/mL. This concentration is significantly lower than the values found in the guinea pig because the marmoset has only a very low concentration of CaE in blood. The concentration of regenerated soman appeared not to be a quantitative marker for inhaled dose. The toxicokinetic curves showed large variation and some marmosets clearly had difficulties breathing during the exposure. Apparently a small amount of soman is sufficient to occupy the available binding sites.

Guinea pig – i.v. toxicokinetics of sarin
The concentration of fluoride regenerated sarin in plasma of naive guinea pigs that were i.v. injected with sarin (48 µg/kg) was 53 ± 14 ng/mL, which corresponds with 379 ± 100 nM. This means that the binding sites (CaE) are not fully occupied if we compare this concentration with the values we found in the soman toxicokinetics. The concentration of regenerated sarin in plasma of HuBuChE-pretreated guinea pigs, which received the same dose of sarin was 379 ± 18 ng/mL, which corresponds with 2707 ± 130 nM. The net increase of regenerated sarin because of HuBuChE treatment is 2707 – 379 nM = 2328 ± 230 nM. The initial BuChE in concentration in plasma was 1300 nM, which is not in agreement with the concentration of regenerated sarin. An explanation for this discrepancy cannot be given. The amount of bound sarin in blood can be estimated as 379 ng/mL * 30 mL/kg = 11 µg. If we suppose that the P(+) isomers are rapidly hydrolyzed, then 24 µg + 11 µg = 35 µg sarin is eliminated in blood.

Guinea pig – inhalation toxicokinetics of sarin
The concentration of fluoride regenerated sarin in plasma of naive guinea pigs that were 2-min nose-only exposed to sarin vapor (200 $mg.m^{-3}$) was 56 ± 6 ng/mL, which corresponds with 403 ± 43 nM. The concentration of regenerated sarin in plasma of HuBuChE-pretreated guinea pigs that were exposed under the same conditions was 241±13 ng/mL which corresponds with 1721 ± 93 nM. The net increase of regenerated sarin is 1721-403= 1318 ± 136 nM. The initial concentration of BuChE in the pretreated animals was 707 nM, which is also not in agreement with the concentration of regenerated sarin.

Hairless guinea pig – VX toxicokinetics
The concentration of fluoride regenerated ethyl sarin in plasma of HuBuChE-pretreated hairless guinea pigs that were i.v. injected with VX (56 µg/kg) was 85 ± 9 ng/mL, which corresponds with 685 ± 73 nM. The initial concentration of HuBuChE was 900 ± 125 nM, which is in reasonable agreement with the concentration of regenerated ethyl sarin.

The concentration of fluoride-regenerated ethyl sarin in plasma of HuBuChE-pretreated hairless guinea pigs which were percutaneously exposed to VX (250 μg/kg) was 15 ± 2 ng/mL, which corresponds with 121 ± 30 nM and is in good agreement with the decrease of the concentration of BuChE in plasma: 152 ± 43 nM. The amount of VX that was scavenged was 121 nM * 30 mL/kg * 267 = 969 ng, which means that almost 1 μg VX had penetrated through the skin into the blood.
The concentration of regenerated ethyl sarin in the naive hairless guinea pigs that were percutaneously exposed to VX (250 μg/kg) was less than 1 ng/mL corresponding with 8 nM. It is supposed that the penetration rate of VX through the skin is not dependent on the presence of BuChE in blood. Therefore it can be assumed that approximately 1 μg VX has penetrated through the skin. However due to slow hydrolysis, the lack of scavenger and other rapid binding places in blood, this 1 μg had the possibility to reach toxicologically relevant organs.

IV.6 Rate constants of inhibition of HuBuChE for nerve agent stereoisomers

The molar concentration of HuBuChE, which is needed for studying the reaction of the enzyme with the potent inhibitors such as (±)-sarin, (±)-VX and C(±)P(±)-soman, could conveniently be derived from the activity of the enzyme by using the relation reported by Grunwald *et al.* (1997) between the activity of HuBuChE expressed as U/mL and its molar concentration.
From the inhibition studies it was concluded that the ratio between the anticholinesterase activities of the two stereoisomers of (±)-sarin and (±)-VX differ only slightly. These results are in correspondence with the general finding that BuChE shows only a minor stereoselectivity for potent chiral organophosphates, in contrast with the high stereoselectivity usually exhibited by AChE for such inhibitors.
The rate constants of inhibition for the two stereoisomers of (±)-sarin and (±)-VX could be evaluated from the reaction of the enzyme with a slightly different concentration of the racemic mixture of the organophosphate by using a kinetic analysis based on a method reported by French (1950). The results obtained for inhibition of HuBuChE are of the same order of magnitude as data previously obtained for horse serum BuChE (see Table 53).

It can be seen that the stereoselectivity of the P(+) and P(-) isomers is not as high as seen for AChE. This means that the binding rate of the relative non toxic P(+)-isomers with BuChE is also considerable. If the P(+)-isomer is slowly eliminated by enzymatic hydrolysis as in the case of (+)-VX this isomer will consume a significant amount of scavenger, which is deleterious for the efficacy of scavenging.

Table 53 Rate constants of inhibition ($M^{-1}.min^{-1}$) of human and horse serum BuChE by the stereoisomers of (±)-sarin, (±)-VX and C(±)P(±)-soman .

Organophosphate	Enzyme source			
	Human		Horse	
Sarin	$4.2 * 10^7$ $5.5 * 10^6$	pH 7.4, 38 °C[a]	$6 * 10^6$ $6 * 10^6$	pH 7.7, 25 °C[b]
VX	$1.3 * 10^7$ $2.5 * 10^6$	pH 7.4, 38 °C[a]	$1.7 * 10^7$	pH 7.7, 25 °C[c]
Soman				
C(+)P(-)	$3.8 * 10^8$	pH 7.4, 38 °C[a]	$8.7 * 10^7$	pH 7.7, 25 °C[d]
C(-)P(-)	$1.2 * 10^7$		$6.4 * 10^6$	
C(-)P(+)	$7.4 * 10^6$		$6.4 * 10^6$	
C(+)P(+)	$2.1 * 10^6$		$2.9 * 10^5$	
C(+)P(-)	$4 * 10^7$	pH 7.5, 25 °C[e]		
C(-)P(-)	$5 * 10^6$			
C(-)P(+)	$6 * 10^6$			
C(+)P(+)	< LOD[f]			

[a] Present study; [b] Boter and Van Dijk (1969); [c] rate constant for racemic mixture, unpublished results of TNO Prins Maurits Laboratory; [d] Keijer and Wolring (1969); [e] Millard *et al.* (1998);
[f] not determined

IV.7 Physiologically-based pharmacokinetic modeling

The concentration-time curves of the intravenous toxicokinetics of C(±)P(-)-soman were predicted using a physiologically-based pharmacokinetic model. The predicted curves were in good agreement with the data that were measured within the framework of DAMD 17-85-G-5004 (6 LD50), DAMD17-87-G-7015 (2 LD50) and DAMD17-95-2-5009 (0.8 LD50). In the current study we measured the i.v. toxicokinetics of soman (55 µg/kg) in naive guinea pigs which should correspond with 2 LD50. The measured concentrations were significantly lower than found in previous work. This topic has been discussed in section IV.2.1. Consequently the measured data do not fit well with the predicted curves obtained from the PBPK model. The model can be used to predict the effect of HuBuChE as scavenger on the toxicokinetics of nerve agents. The AUC proved to be a good parameter to study this effect. Table 54 shows the calculated AUCs of the modeled toxicokinetic curves.

Table 54 AUC of modeled i.v. toxicokinetics of soman with and without HuBuChE pretreatment

Toxicity equivalent	Dose (µg/kg) C(±)P(±)-soman	HuBuChE pretreatment	AUC (ng.min.mL^{-1}) C(+)P(-)-soman	AUC (ng.min.mL^{-1}) C(-P(-)-soman
0.8 LD50	22	no, see Table 41	14	22
0.8 LD50	22	yes, see Table 42	1.7	6.7
2LD50	55	no, see Table 41	143	172
2LD50	55	yes, see Table 42	25	88
6LD50	165	no, see Table 41	633	578
6LD50	165	yes, see Table 42	471	560

The decrease in AUC of C(±)P(-)-soman isomers in the 6 LD50 experiments as result of HuBuChE pretreatment is (633+578-471-560) = 180 ng.min.mL^{-1} which corresponds with 1.0 nmol.min.mL^{-1}. When naive guinea pigs are i.v. injected with a high dose of soman all binding sites will be occupied. Additional binding sites, *i.e.* BuChE will be all consumed and the result is a lower AUC.
The decrease in AUC of C(±)P(-)-soman isomers in the 2 LD50 experiments as result of HuBuChE pretreatment is (143+172-25-88) = 202 ng.min.mL^{-1} which corresponds with 1.1 nmol.min.mL^{-1}. The balance between rapid binding sites and soman is still in favor of soman, which means that additional rapid binding sites will be consumed.
The decrease in AUC of C(±)P(-)-soman isomers in the 0.8 LD50 experiments as result of HuBuChE pretreatment is (14+22-1.7-6.7) = 27.6 ng.min.mL^{-1} which corresponds with 0.15 nmol.min.mL^{-1}. The balance between binding sites and soman is in favor of the binding sites. If additional rapid binding sites become available, they will not be consumed all.

The decrease in AUC of C(±)P(-)-soman isomers in the actual toxicokinetic experiments as a result of HuBuChE pretreatment was 63 ng.min.mL^{-1} which corresponds with 0.34 nmol.min.mL^{-1}. This is an indication that the administered dose of soman was lower than intended or that the amount of available binding sites was higher than the number that was used as input for the model.

The model predicts also the concentrations of soman, free AChE, BuChE and CaE in the various organs. The curves pertaining to the concentration of soman vs. time are shown in section III.13. The liver is the only organ that is treated as a separate organ in this model. The calculations showed that free AChE, BuChE and CaE in blood were completely absent after i.v. injection of soman. The concentrations of the enzymes in the liver are shown in Table 55. The concentration of BuChE in HuBuChE-pretreated animals after i.v. injection of soman corresponding with 2 LD50 was 52 nM, which is 26% of the initial amount. The residual BuChE concentration in the liver is 0.85 nM in naive animals that received the same dose of soman. The blood flow to the liver is only 0.45 mL/min, which is a limiting factor for the amount of soman that enters the liver and limits the decrease of BuChE and CaE concentration in the liver.
If the dose of soman is higher (110 µg/kg) the residual free BuChE concentration decreased to zero. The concentration of CaE decreases only slightly more than after the injection of the lower soman dose. The large amount of CaE is barely occupied because of the flow limitation.
The residual BuChE concentration in HuBuChE-pretreated animals that were i.v. injected with 110 µg/kg of soman was 25% of the initial concentration (see section III.12). This result is not in agreement with the predictions of the model. However this result verifies that a significant residual BuChE concentration will be present in the liver after injection of soman corresponding with multiple LD50s.

Table 55 Calculated concentration (nM) of free BuChE and CaE in liver of naive and HuBuChE-pretreated guinea pigs which were challenged with various doses of soman

Soman(μg/kg)	55	55	55	55	110	110	110	110
HuBuChE	yes, see Table 42		no, see Table 42		yes, see Table 42		no, see Table 41	
Time (min)	BuChE	CaE	BuChE	CaE	BuChE	CaE	BuChE	CaE
0	85	20,000	85	20,000	85	20,000	85	20,000
5	31.5	19,400	4.25	19,400	0.85	19,200	85	20,000
10	29.8	19,400	1.7	19,000	0	18,800	0.85	19,000
15	27.2	19,400	0.85	19,000	0	18,400	0	18,000
20	25.5	19,400	0.85	18,800	0	18,000	0	17,400
40	23.8	19,400	0.85	18,600	0	17,400	0	16,600

IV.8 Some comments concerning the animal experiments

The toxicokinetics of soman in the current study were studied with guinea pigs that were purchased from Harlan. The toxicokinetics of soman that were studied in 1989 in animals that were purchased from Charles River. During this study it appeared that the AUC of the toxicokinetic curves of soman were 5-fold lower than the AUC of the toxicokinetic curves that were measured in 1989. It must be realized that guinea pigs are outbred species, which means that differences in toxicokinetics in the course of time can easily occur.

During this study it appeared that an abnormal high percentage of the animals died before the start of the toxicokinetic experiment. At 24 h after HuBuChE administration, the normal looking animals were anesthetized and at that time a significant number of animals died. After several incidents it was decided to perform necropsy on all animals that were used in animal experiments. A total of 18 animals were investigated. Five animals died prematurely after receiving anesthesia. Six animals survived the experiment but showed severe signs upon necropsy. The signs were hemorrhage and clotted blood in the belly. Two animals survived the experiments but showed mild signs during necropsy, such as a glazed kidneys and bleedings for various organs upon manipulation with tweezers. Five animals showed no symptoms. Control animals (n=2) showed no severe signs.

HuBuChE-pretreated marmosets 9 and 10 died after being anesthetized. With the above mentioned experience with the guinea pigs in mind, it could not be excluded that there might be a side effect of HuBuChE. It was decided to stop the experiments with marmosets, because we did not want to sacrifice more marmosets without having a reasonable chance to obtain valuable results.

In the last year of the grant period 13 hairless guinea pigs were i.m. injected with HuBuChE. One animal died after being anesthetized and showed bleedings in the belly. Six animals showed mild signs, such as glazed kidneys. Five animals had no symptoms.

In the last year of the grant period six guinea pigs were i.m. injected with HuBuChE. The first animal died after being anesthetized. The other animals died during the experiment, because of the soman intoxication but showed mild signs in the necropsy.

Sofar side-effects due to administration of HuBuChE have never been reported. However it should be mentioned that this kind of experiment, (autopsy at 24 h after i.m. injection of the enzyme) was never performed before. It is conceivable that increasing the concentration of BuChE in blood to extraordinary high levels may lead to temporarily critical conditions in the vascular system. Upon our request colleagues at the USAMRICD performed the same experiments in December 2003. Guinea pigs were i.m. injected with HuBuChE and at 24 h after

the i.m. injection examined in a necropsy. They did not find any abnormalities at all (n=3). There was not enough enzyme available to perform a full study with regard to the possible side effects of HuBuChE. Such a study was also beyond the scope of this study. Thirdly, the number of negative control animals was also too small to draw conclusions about the safety of HuBuChE. Nevertheless it must be kept in mind that administration of HuBuChE might lead to some side effects. Perhaps it is advisable to perform a study on the side-effects of HuBuChE in which HuBuChE-pretreated animals (i.m. injection) and negative control animals will be examined by means of necropsy at the time points at which the maximum BuChE concentration in blood is reached.

V KEY RESEARCH ACCOMPLISHMENTS

Intramuscular administration of HuBuChE to guinea pigs and marmosets leads to a gradual build-up of BuChE activity in blood.

The maximum concentration HuBuChE in blood of guinea pigs was reached at 24 h after i.m. administration of HuBuChE.

A total of approximately 20 -30 % the i.m. administered dose HuBuChE circulated in blood of guinea pigs, respectively at 24 h after i.m. administration.

The maximum concentration HuBuChE in blood of marmosets was reached at 12 h after i.m. administration of HuBuChE.

Approximately 13-16 % of the i.m. administered dose HuBuChE circulated in blood of marmosets at 24 h after i.m. injection.

A total of approximately 3 % of the i.m. administered dose of HuBuChE was recovered in the extravascular compartments of liver, kidney, lungs, and brain at 24 h after i.m. administration, which means that the fate of 67% of the amount of administered enzyme is unknown.

The residual BuChE activity in blood of HuBuChE-pretreated guinea pigs (200 nmol/kg) after i.v. administration of soman (604 nmol/kg) was negligible.

Approximately 25% of the initial BuChE activity in liver of HuBuChE-pretreated guinea pigs (200 nmol/kg) remained intact after i.v. injection of soman (604 nmol/kg).

The residual area-under-the-curve (AUC) of nerve agent in blood appeared to be a good indicator for the efficacy of HuBuChE as a scavenger.

The AUC of (-)-sarin in blood of guinea pigs after i.v. administration of a dose of (±)-sarin (48 μg/kg ≡ 342 nmo/kg) corresponding with 2 LD50, was 109 ng.min.mL^{-1}. This AUC decreased to 2.4- 29 ng.min.mL^{-1}as a result of pretreatment with 200 nmol HuBuChE /kg.

Remarkably, the concentrations of (-)-sarin in blood of guinea pigs appear to level off or even increase in the terminal phase. This phenomenon seems not to occur with C(±)P(-)-soman and VX.

The AUC of C(±)P(-)-soman in blood of guinea pigs after i.v. injection of a dose of C(±)P(±)-soman (55 μg/kg ≡ 302 nmol/kg), corresponding with 2 LD50, was 65.9 ng.min.mL^{-1}. This AUC decreased to 2.9 ng.min.mL^{-1} as a result of pretreatment with HuBuChE (200 nmol/kg).

The AUC of C(±)P(-)-soman in blood of guinea pigs after i.v. injection of two doses of C(±)P(±)-soman (55 μg/kg), corresponding with 2LD50, in HuBuChE-pretreated guinea pigs, was 176 ng.min.mL^{-1}.

The AUC of (±)-VX in blood of hairless guinea pigs after i.v. injection of a dose of (±)-VX (56 μg/kg ≡ 205 nmol/kg), corresponding with 2 LD50 was 765 ng.min.mL^{-1}. This AUC decreased to 494 ng.min.mL^{-1} as a result of pretreatment with HuBuChE (200 nmol/kg).

The AUC of (-)-sarin in blood after 2-min nose-only exposure to (±)-sarin vapor (200 $mg.m^{-3}$) ranged between 7 and 61 $ng.min.mL^{-1}$. This AUC decreased to 4-22 $ng.min.mL^{-1}$ as a result of pretreatment with HuBuChE (100 nmol/kg).

The concentration of sarin in blood of guinea pigs that were exposed for 2 min to sarin vapor (200 $mg.m^{-3}$) increased until 4 min after the start of the exposure. This phenomenon was not observed with soman.

The AUC of C(±)P(-)-soman in blood of guinea pigs after a 2-min nose-only exposure to C(±)P(±)-soman vapor (200 $mg.m^{-3}$) was 22 $ng.min.mL^{-1}$. This AUC decreased to 0.3- 6 $ng.min.mL^{-1}$ as a result of pretreatment with HuBuChE (100 nmol/kg).

A guinea pig that was continuously nose-only exposed to C(±)P(±)-soman vapor (2 $mg.m^{-3}$), died after 2-3 h exposure time. Under the same conditions, HuBuChE-pretreated animals (100 nmol/kg) survived at least one h longer although having inhaled a larger internal dose of C(±)P(±)-soman as verified by the fluoride reactivation assay.

The AUC of C(±)P(-)-soman in blood of marmosets after a 5-min nose-only exposure to C(±)P(±)-soman vapor (50 $mg.m^{-3}$) ranged between 6.7 and 54.2 $ng.min.mL^{-1}$. This AUC decreased to 0 - 6 $ng.min.mL^{-1}$ as a result of pretreatment with HuBuChE (± 85 nmol/kg).

The AUC of (±)-VX in blood of HuBuChE-pretreated hairless guinea pigs (100 nmol/kg) which were percutaneously exposed to (±)-VX (250 µg/kg) was negligible.

The fluoride reactivation method is a valuable tool for the verification of internal dosages in guinea pigs after respiratory or percutaneous exposure, to quantify the number of occupied binding sites and to demonstrate the efficacy of BuChE as scavenger for regenerable nerve agents, such as sarin and VX.

A physiologically-based pharmacokinetic model was developed for the toxicokinetics of soman. The predictions of the model are in reasonable agreement with the data actually measured in animals.

HuBuChE shows only a minor stereoselectivity for the stereoisomers of chiral nerve agents, in contrast with the high stereoselectivity exhibited by AChE towards these anticholinesterases.

The data obtained for inhibition rate constants of HuBuChE with the enantiomers of nerve agent are of the same order of magnitude as those previously reported for horse serum BuChE.

VI REPORTABLE OUTCOMES

Publications

VAN DER SCHANS, M.J., VAN DER WIEL, H.J., SPRUIT, W.E.T., TRAP, H.C., VAN DIJK, C., VAN DER LAAKEN, A.L., PLEIJSIER, K., NOORT, D., DE JONG, L.P.A., BENSCHOP, H.P. AND LANGENBERG, J.P. (2002) Effect of pretreatment with human butyrylcholinesterase scavengers on the toxicokinetics and binding of nerve agents in guinea pigs. Proceedings of the 2002 Medical Chemical Bioscience Review, June 3-7, 2002, Hunt Valley (MD), USA

VAN DER SCHANS, M.J., PLEIJSIER, K., VAN DER WIEL, H.J., SPRUIT, W.E.T. AND LANGENBERG, J.P., (2002) Effect of pretreatment with Human Butyrylcholinesterase scavengers on the toxicokinetics and binding of nerve agents in guinea pigs. Presentation at NATO-TG004 meeting Oslo, Norway, November 4-7, 2002

VAN DER SCHANS, M.J., LANGENBERG, J.P. AND BENSCHOP, H.P. (2004) Toxicokinetics and Binding of Nerve agents in the presence of scavengers. Proceedings at 2004 Medical Chemical Bioscience Review, May 16-21, 2004, Hunt Valley (MD).

VAN DER SCHANS, M.J., PLEIJSIER, K., BOONE, C.M., VAN DER WIEL, H., LANGENBERG, J.P. AND BENSCHOP, H.P. (2004) Effect of pretreatment with human butyrylcholinesterase scavengers on the toxicokinetics and binding of nerve agents, Proceedings at 2004 Medical Chemical Bioscience Review, May 16-21, 2004, Hunt Valley (MD).

Presentations

VAN DER SCHANS, M.J., VAN DER WIEL, H.J., SPRUIT, W.E.T., TRAP, H.C., VAN DIJK, C., VAN DER LAAKEN, A.L., PLEIJSIER, K., NOORT, D., DE JONG, L.P.A., BENSCHOP, H.P. AND LANGENBERG, J.P. (2002) Effect of pretreatment with human butyrylcholinesterase scavengers on the toxicokinetics and binding of nerve agents in guinea pigs. Poster presented at the 2002 Medical Chemical Bioscience Review, June 3-7, 2002, Hunt Valley (MD).

VAN DER SCHANS, M.J., PLEIJSIER, K., VAN DER WIEL, H.J., SPRUIT, W.E.T. AND LANGENBERG, J.P., (2002) Effect of pretreatment with Human Butyrylcholinesterase scavengers on the toxicokinetics and binding of nerve agents in guinea pigs. Presentation at NATO-TG004 meeting Oslo, Norway, November 4-7, 2002

VAN DER SCHANS, M.J., LANGENBERG, J.P. AND BENSCHOP, H.P. (2004) Toxicokinetics and Binding of Nerve agents in the presence of scavengers. Presentation at 2004 Medical Chemical Bioscience Review, May 16-21, 2004, Hunt Valley (MD).

VAN DER SCHANS, M.J., PLEIJSIER, K., BOONE, C.M., VAN DER WIEL, H., LANGENBERG, J.P. AND BENSCHOP, H.P. (2004) Effect of pretreatment with human butyrylcholinesterase scavengers on the toxicokinetics and binding of nerve agents, Poster presented at 2004 Medical Chemical Bioscience Review, May 16-21, 2004, Hunt Valley (MD).

VII CONCLUSION

Human butyrylcholinesterase is a drug candidate for protection against organophosphate intoxication. BuChE is a stoichiometric scavenger which means that the scavenger is consumed when it reacts with a nerve agent molecule. A sufficient concentration of BuChE in blood will lead to an efficient elimination of nerve agent in order to reduce the possibility that the nerve agent will reach essential organs such as the diaphragm and the brains. The advantages of BuChE as antidote are the extremely high reactivity towards nerve agents and the preference for binding with the most toxic stereoisomer of the nerve agent. Catalytic scavengers often do not react that rapidly with the nerve agents and have mostly a preference for reaction with the non-toxic stereoisomer (Hill *et al*, 2001).
Several researchers have developed mathematical models to calculate the amount of stoichiometric scavenger that is needed to neutralize a certain amount of nerve agent (Sweeney and Maxwell, 1999, 2003, Ashani and Pistinner, 2003). Their calculations are based on the binding constants of nerve agent with AChE and BuChE and the concentrations of the enzymes and nerve agent. A sufficient protection is achieved when the residual AChE concentration remains above a level of 30 % inhibition. They also propose that the reactions take place in one compartment. However, when HuBuChE will be used in a military setting, it is likely that the enzyme will be administered by an i.m. injection using an auto-injector device. The i.m. administration of the enzyme will lead to a gradual build up of the concentration BuChE in blood. In our study the maximum concentration of BuChE in blood of guinea pigs was reached at 24 h after i.m. administration, whereas in marmosets the maximum concentration was reached at 12 h after i.m. administration. Other researchers found a Tmax within 12 h in rhesus monkeys, rats and mice. A finding of concern, however, was that only 20-30 % of the amount of administered enzyme was recovered in blood at Tmax. This rather low recovery will have negative consequences for the efficacy of the scavenger. The i.m. administration of the scavenger is also an important deviation from the protocol that was followed by other researchers (Ashani, 1993, Raveh *et al*, 1997, Allon *et al*, 1998). They administered the enzyme i.v. and challenged the animals with nerve agent not later than 90 min after administration of the enzyme. By the latter protocol the concentrations of enzyme in blood were very high, which resulted in impressive protection.

The dose of i.m. administered HuBuChE was 0.7 times the dose of the nerve agent on a molar basis. The nerve agent was i.v. administered at 24 h after the injection of the enzyme. In a best guess it can be estimated that the dose of nerve agent that will remain corresponds with (1-0.7) *2 LD50 = 0.6 LD50.
The toxicokinetics of (±)-VX are rather linear and the elimination process is mainly described by distribution and slow enzymatic hydrolysis. The AUC appeared to be a valuable tool for comparison of the toxicokinetics in naive and HuBuChE pretreated animals. Upon pretreatment with HuBuChE the AUC of the i.v. toxicokinetics of (±)-VX was reduced with 270 ng.min.mL^{-1}, *i.e.,* a disappointing reduction with only 35%. The time period in which acutely toxic levels of VX circulated was reduced from 310 to 230 min. The value of the AUC was approximately the same as that expected after an i.v. administration of VX corresponding with 1.5 LD50. A reason for this smaller than expected effect must be found in the availability of the scavenger. Only 30 % of the scavenger was recovered in blood and another 3 % of the scavenger was recovered in organs such as liver, lung, kidney and brain. The effect of HuBuChE treatment on the toxicokinetics of soman is more difficult to interpret. The elimination of soman is caused by distribution, rapid enzymatic hydrolysis but also by binding to endogenous rapidly binding sites

such as CaE. Therefore (the linearity) of the toxicokinetics soman must be divided in a low dose (non saturated CaE) and high dose (saturated CaE) region. Upon treatment with HuBuChE (200 nmol/kg) the AUC of the toxicokinetics of soman was reduced with 63 ng.min.mL^{-1} (reduction of 82 %). On a molar basis the reduction of the AUC of soman (0.35 nmol.min.mL^{-1}) was less than for VX (0.79 nmol.min.mL^{-1}). The time period in which acutely toxic levels of soman circulate was reduced from 40 min to less than 1 min. This spectacular result is obtained because the dose of soman was reduced by the scavenger to a dose that is not sufficient to saturate all endogenous rapidly reacting binding sites. Toxicokinetic studies in the past revealed that doses of nerve agent lower than the amount of available rapidly binding sites showed a low AUC of the toxicokinetic curve. The existence of additional binding sites such as CaE hampers a clear interpretation of the scavenging effect of HuBuChE on the toxicokinetics. Certainly, extrapolation of the results to humans will become problematic. Perhaps it would have been better to study the effect of the scavenger at higher doses of soman in order to reduce the role of endogenous rapidly binding sites.
In principle the toxicokinetics of sarin are governed by the same phenomena as mentioned for soman but are even more difficult to interpret because they were characterized by a long terminal half life. The effect of the scavenger on the AUC was evident: upon treatment with HuBuChE (200 nmol/kg) the AUC of an i.v. dose of 48 µg/kg (343 nmol/kg) was reduced with 80 ng.min.mL^{-1} (reduction of 73%), but sarin appeared to be rather persistent, also in HuBuChE-pretreated animals. It seems that there exists an equilibrium between free and non-covalently bound sarin. This phenomenon was also observed by Spruit *et al*, (2000). It would be interesting to study the toxicokinetics of sarin in a species with a smaller amount of rapidly binding sites in blood, e.g. marmosets. If the levels of sarin appear to be persistent also in marmosets, then these levels might cause considerable complications for therapy and prophylaxis. The therapy with oximes of sarin-intoxicated animals never appeared to be problematic, because sarin-inhibited AChE is rapidly reactivated by oximes. However, the persistent levels of sarin might become critical, if the casualty is only protected by prophylaxis with carbamates.

HuBuChE as pretreatment was also studied using a more realistic porte d'entrée, *i.e.,* the respiratory route in which the nerve agent entered more gradually. Naive and HuBuChE-pretreated guinea pigs were 2-min nose-only exposed to soman and sarin vapor in air (400 mg.min.m^{-3}). The AUC of the toxicokinetics of HuBuChE (100 nmol/kg)-pretreated guinea pigs was significantly lower than that in naive animals. In some cases soman could not even be detected, indicating complete scavenging of the nerve agent. Unfortunately, a 2-min exposure to high vapor concentrations appeared to be rather irreproducible. When toxicokinetics are to be studied it is preferable to expose the animals for 8-min in order to create exposure conditions that can be better controlled, albeit that a 2-min exposure scenario is more realistic.
In another series of experiments in which guinea pigs were exposed to a low level of soman (2 mg.m^{-3}) vapor in air for 300 min, the majority of the animals did not survive the experiment because of soman intoxication. However, the HuBuChE-pretreated animals lived at least one hour longer than the negative control animal (n=1), while it was verified by means of the fluoride reactivation method that the HuBuChE-pretreated animals had obtained a higher internal dose of soman.
The respiratory toxicokinetics of sarin in naive and HuBuChE-pretreated guinea pigs were difficult to interpret. The average AUC of the toxicokinetics of HuBuChE-pretreated animals was indeed lower but the spread was such large that there was overlap between the naive and pretreated groups.
The interfering effect of CaE on the toxicokinetics is less apparent in marmosets. Because of the higher toxicity of soman in marmosets, the animals were nose-only exposed to a lower Ct value

(Ct 250 mg.min.m^{-3}). The AUC of the toxicokinetics of soman in HuBuChE-pretreated marmosets (71 nmol/kg) decreased 0.7- 48 ng.min.mL^{-1} , *i.e.*, a reduction of 10- 88%.

Finally, HuBuChE-pretreated hairless guinea pigs were percutaneously exposed to a dose of VX corresponding with 2 LD50. Intact VX was not detected, which is not unexpected because the penetration of VX through the skin proceeds very slowly. Actually, the percutaneous toxicokinetics of VX should be measured more thoroughly and for a longer time period after exposure.

It is obvious that the availability of an adequate PBPK model would be highly valuable because it allows to study the influence of binding sites (CaE or scavenger) on the toxicokinetics of nerve agents with more variables than ever can be performed with animal experiments. We have a PBPK model available for the i.v. toxicokinetics of soman, but models for VX and other additional administration routes are also needed.

Pretreatment with HuBuChE is effective in the reduction of the AUC of the toxicokinetics of nerve agents. However, a stoichiometric scavenger will be consumed upon reaction with nerve agents, which means that the required dosage of enzyme and nerve agent challenge are related to each other. Within this context it would be interesting to study the toxicokinetics of nerve agents in the presence of both HuBuChE and oximes, which would create a semi-catalytic highly reactive scavenger for nerve agents. Secondly, it is essential that the scavenger circulates in blood at a high concentration at the time of the exposure. It is questionable whether this requirement can be met with an i.m. administration of the enzyme, since the systemic bioavailability was only 20-30 % at Tmax. Pharmacokinetic studies with human volunteers should give more insight into the recovery of enzyme in blood after i.m. administrations, provided that the material is safe and causes no side-effects. Supporting in this respect would be further animal studies focusing on the distribution of the enzyme over the organs following i.v. administration in order to investigate the recovery of the enzyme after a residence time of 24 h. An i.v. administration of the enzyme would overcome the problem of the low recovery of the enzyme in blood after i.m. injection. However an i.v. injection can only be administered by medically trained personnel.
In view of the above mentioned results, it would be relevant to investigate alternative routes for administering HuBuChE in a more efficient way that is either by means of an aerosol via the respiratory route, and/or via the oral route (mouth and esophagus) using low surface tension solutions. Considering the large absorption surface of both routes, compared to i.m. injection, a more rapid and more complete uptake of the enzyme may be expected. Moreover, the scavenger would be present at the most likely porte d'entrée of a nerve agent. Another application of HuBuChE may be found in i.m. injected HuBuChE as a *therapeutic* agent against slowly penetrating nerve agents. It is expected that the absorption of enzyme after i.m. injection proceeds more rapidly than the penetration of VX through the skin.

VIII REFERENCES

Allon, N., Raveh, L., Gilat, E., Cohen, E., Grunwald, J., and Ashani, Y. (1998) Prophylaxis against soman inhalation toxicity in guinea pigs by pretreatment alone with human serum butyrylcholinesterase. *Toxicol. Sci.* **43**, 121-128.

Ashani, Y., Shapira, S., Levy, D., Wolfe, A.D., Doctor, B.P., and Raveh, L. (1991) Butyrylcholinesterase and acetylcholinesterase prophylaxis against soman poisoning in mice. *Biochem. Pharmacol.* **41**, 37-41.

Ashani, Y., Grunwald, J., Raveh, L., Grauer, E., Brandeis, R.., Marcus, D., Papier, Y., Kadar, T., Allon, N., Gilat, E., and Lerer, S. (1993) Cholinesterase prophylaxis against organophosphorus poisoning. Final Report Contract DAMD17-90-C-0033; AD-A277-096.

Ashani, Y.,(2000) Prospective of human butyrylcholinesterase as detoxifying antidote and potential regulator of controlled-release drugs, *Drug Dev. Res.,* **50**, 298-308.

Ashani, Y. and Pistinner, S. (2004) Estimation of the upper limit of human butyrylcholinesterase dose required for protection against organophosphates toxicity: a mathematically based toxicokinetic model, *Toxicol. Sci.* **77**, 358-367.

Benschop, H.P., and De Jong, L.P.A. (1987) Toxicokinetics of the four stereoisomers of soman in the rat, guinea pig and marmoset, Final Report Grant DAMD17-85-G-5004.

Benschop, H.P., and De Jong, L.P.A. (1990) Toxicokinetic Investigations of C(±)P(±)-soman in the rat, guinea pig and marmoset at low dosages – Quantification of elimination pathways, Final report DAMD17-87-G-7015.

Benschop, H.P., and De Jong, L.P.A. (1988) Nerve agent stereoisomers: analysis, isolation, and toxicology. *Acc. Chem. Res.* **21**, 368-374.

Benschop, H.P., and De Jong, L.P.A. (1991) Toxicokinetics of soman: Species variation and stereospecificity in elimination pathways. *Neurosci. Biobehav. Rev.* **15**, 73-77.

Benschop, H.P. and Van Helden, H.P.M. (1993) Toxicokinetics of inhaled soman and sarin in guinea pigs. Final Report Grant DAMD17-90-Z-0034.

Benschop, H.P., Konings, C.A.G., Van Genderen, J., and De Jong, L.P.A. (1984) Isolation, anticholinesterase properties, and acute toxicity in mice of the four stereoisomers of the nerve agent soman. *Toxicol. Appl. Pharmacol.* **72**, 61-74.

Benschop, H.P., Bijleveld,. E.C., De Jong, L.P.A., Van der Wiel, H.J., and Van Helden, H.P.M. (1987) Toxicokinetics of the four stereoisomers of the nerve agent soman in atropinized rats – Influence of a soman simulator. *Toxicol. Appl. Pharmacol.* **90**, 490-500.

Benschop, H.P., De Jong, L.P.A., and Langenberg, J.P. (1995) Inhalation toxicokinetics of C(±)P(±)-soman and (±)-sarin in the guinea pig. In: *Enzymes of the Cholinesterase Family* (Quin, D.M., Balasubramian, A.S., Doctor, B.P., and Taylor, P., Eds). Plenum Press, pp. 361-368.

Benschop, H.P., Trap, H.C., Spruit, W.E.T., Van der Wiel, H.J., Langenberg, J.P. and De Jong, L.P.A. (1998) Low level nose-only exposure to the nerve agent soman: toxicokinetics of soman stereoisomers and cholinesterase inhibition in atropinized guinea pigs. *Toxicol. Appl. Pharmacol.* **153**, 179-185..

Black, R.M., and Harrison, J.M. (1996) The chemistry of organophosphorus chemical warfare agents, in: *The chemistry of organophosphorus compounds, Vol. 4* (Hartley, F.R., Ed.), Chapter 10. John Wiley & Sons Ltd, Chichester, pp. 781-840.

Bonierbale, E., Debordes, L. and Coppet, L. (1996) Application of capillary gas chromatography to the study of the hydrolysis of the nerve agent VX in rat plasma. *J. Chromatogr.*, **668**, 255-264.

Bosse, J.A. and Wasserman, (1970) On the blood content of guinea pig tissues, *Pharmacol.*, **4**, 273-277

Boter, H.L., and Van Dijk, C. (1969) Stereospecificity of hydrolytic enzymes on reaction with asymmetric organophosphorus compounds. III. The inhibition of acetylcholinesterase and butyrylcholinesterase with enantiomeric forms of sarin. *Biochem. Pharmacol.* **18**, 2403-2407.

Brimfield, A.A., Hunter, Jr., K.W., Lenz, D.E., Benschop, H.P., Van Dijk, C., and De Jong, L.P.A. (1985) Structural and stereochemical specificity of mouse monoclonal antibodies to the organophosphorus cholinesterase inhibitor soman. *Mol. Pharmacol.* **28**, 32-39.

Broomfield, C.A., Maxwell, D.M., Solana, R.P., Castro, C.A., Finger, A.V., Lenz, D.E., (1991) Protection by butyrylcholinesterase against organophosphorus poisoning in non human primates, *J. Pharm.Exp. Therap.*, **259**, 633-638.

Cohen, J.A., and Warringa, M.G.P.J. (1957) Purification and properties of dialkylfluorophosphates. *Biochim. Biophys. Acta* **26**, 29-39.

Cresthull, P., Koon, W.S., and Oberst, F.W. (1957) Inhalation effects (incapacitation and mortality) for monkeys exposed to GA GB and GF vapors. CWLR 2179, U.S. Army Chemical Warfare Laboratories Technical Report, Army Chemical Center, MD.

De Jong, L.P.A. (1993) Determination of parameters for development of a physiologically-based model for the toxicokinetics of C(±)P(±)-soman. Final Report for Cooperative Agreement DAMD17-92-V-2004.

De Jong, L.P.A. (1996) Physiologically-based modeling of C(±)P(±)-soman toxicokinetics in guinea pig. Final Report for Cooperative Agreement DAMD17-95-2-5009.

De Jong, L.P.A., Sweeney, R.E., Maxwell, D.M., Langenberg, J.P., and Benschop, H.P. (2000) A physiologically based model for the toxicokinetics of C(±)P(±)-soman in the atropinized guinea pig and marmoset - Effect of scavengers and hydrolytic enzymes promoting elimination. In: *Proceedings of the 2000 U.S. Army Medical Defense Bioscience Review*, 4-6 June 2000, Hunt Valley (MD), pp. 98-107.

De La Hoz, D., Doctor, B.P., Ralston, J.S., Rush, R.S., and Wolfe, A.D. (1986) A simplified procedure for the purification of large quantities of fetal bovine serum acetylcholinesterase. *Life Sci.* **39**, 195-199.

Doctor, B., Blick, D.W., Caranto, G., Castro, C.A., Gentry, M.K., Larrsion, R., Maxwell, D.M., Murphy, M.R., Schutz, M., Waibel, K. and Wolfe, A.D., (1993) Cholinesterase as scavengers for organophosphorus compounds: protection of primate performance against soman toxicity, *Chem. Biol. Interact.* **87**, 285-293.

Due, A.H., Trap, H.C., Van der Wiel, H.J., and Benschop, H.P. (1993) The effect of pretreatment with CBDP on the toxicokinetics of soman stereoisomers in rats and guinea pigs. *Arch. Toxicol.* **67**, 706-711.

Due, A.H., Trap, H.C., Langenberg, J.P., and Benschop, H.P. (1994) Toxicokinetics of soman stereoisomers after subcutaneous administration to atropinized guinea pigs. *Arch. Toxicol.* **68**, 60-63.

Ellman G.L., Courtney K.D., Andres V. and Featherstone, R.M. (1961), A new rapid colorimetric determination of acetylcholinesterase activity, *Biochemical Pharmacology*, **7**: 88-95.

Fleisher, J.H., Harris, L.W. (1965) Dealkylation as a mechanism for ageing of cholinesterase after poisoning with pinacolyl methylphosphonofluoridate. *Biochem. Pharmacol.* **14**, 641-650.

French, D. (1950) Kinetic analysis of consecutive irreversible second order reactions. *J. Am. Chem. Soc.* **72**, 4806-4807.

Grunwald, J., Marcus, D., Papier, Y, Raveh, L., Pittel, Z., and Ashani, Y. (1997) Large-scale purification and long-term stability of human butyrylcholinesterase: a potential biocsavenger drug, *J. Biochem. Biophys. Methods,* **34**, 123-135.

Harris, L.W., Braswell, L.M., Fleisher, J.H., and Cliff, W.J. (1964) Metabolites of pinacolyl methylphosphonofluoridate (soman) after enzymatic hydrolysis *in vitro*. *Biochem. Pharmacol.* **13**, 1129-1136.

Hill, C.M., Li, W.S., Cheng, T.C., DeFrank, J.J. and Raushel, F.M., (2001) Stereochemical specificity of organophosphorus acid anhydrolase towards p-nitrophenyl analogs of soman and sarin, *Bioorg. Chem.*, **29**, 27-35.

Johnson, C.D., and Russell, R.L. (1975) A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. *Anal. Biochem.* **64**, 229-238.

Keijer, J.H., and Wolring, G.Z. (1969) Stereospecific aging of phosphonylated cholinesterases. *Biochim. Biophys. Acta* **185**, 465-468.

Langenberg, J.P., Van Dijk, C., Sweeney, R.E., Maxwell, D.M., De Jong, L.P.A., and Benschop, H.P. (1997) Development of a physiologically based model for the toxicokinetics of C(±)P(±)-soman in the atropinized guinea pig. *Arch. Toxicol.* **71**, 320-331.

Langenberg, J.P., van der Schans, G.P., Spruit, H.E., Kuijpers, W.C., Mars-Groenendijk, R.H., van Dijk-Knijnenburg, H.C., Trap, H.C., van Helden, H.P., Benschop, H.P., (1998), Toxicokinetics of sulfur mustard and its DNA-adducts in the hairless guinea pig, *Drug Chem. Toxicol.* **21** Suppl 1:131-47.

Langenberg, J.P., Benschop, H.P., and Van der Schans, G.P., (1998a). Toxicokinetics of sulfur mustard and its DNA-adducts in the hairless guinea pig- DNA-adducts as a measure for epithelial damage. Final report Grant No DAMD17-94-V-4009.

Langenberg, J.P., Spruit, W.E.T., Van der Wiel, H.J., Trap, H.C., Helmich, R.B., Bergers, W.W.A., Van Helden, H.P.M., and Benschop, H.P. (1998b) Inhalation toxicokinetics of soman stereoisomers in the atropinized guinea pig with nose-only exposure to soman vapor. *Toxicol. Appl. Pharmacol.* **151**, 79-87.

Lenz, D.E., Brimfield, A.A., Hunter, Jr., K.W., Benschop, H.P., De Jong, L.P.A., Van Dijk, C., and Clow, T.R. (1984) Studies using a monoclonal antibody against soman. *Fundam. Appl. Toxicol.* **4**, S156-S164.

Lenz, D.E. (2004) Bioscavenger Program Review, Presentation at International Medical Chemical Defence Conference, Munich, Germany, April 27, 2004

Marrs, T.C., Maynard R.L., and Sidell, F.R. (1996) Chemical Warfare Agents. Toxicology and Treatment. John Wiley & Sons Ltd, Chichester.

Maxwell, D.M., Castro, C.A., De La Hoz, D., Gentry, M.K., Gold, M.B., Solana, R.P., Wolfe, A.D., and Doctor, B.P. (1992) Protection of rhesus monkeys against soman and prevention of performance decrement by pretreatment with acetylcholinesterase. *Toxicol. Appl. Pharmacol.* **115**, 44-49.

Maxwell, D.M., (1992) The specificity of carboxylesterase protection against the toxicity of organophosphorus compounds, *Toxicol. Appl. Pharmacol.* **114**, 306-312.

Maxwell, D.M., Saxena, A., Gordon, R.K., and Doctor B.P., (1999) Improvements in scavenger protection against organophosphorus agents by modification of cholinesterases, *Chem-Bio Interactions,* **119-120**, 419-428.

Millard, C.B., Lockridge, O., and Broomfield, C.A. (1998) Organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase: synergy results in a somanase. *Biochemistry* **37**, 237-247.

Pearce, P., Passingham, S., Scott, L., Busker, R., and Van Xanten, N. (1999) A comparison of oximes, when combined with atropine and Avizafone, in the treatment of nerve agent poisoning in absence of pretreatment. Abstract Book of the Meeting of NATO TG004 in Brussels, March 22-26, 1999.

Polhuijs, M., Langenberg, J.P., and Benschop, H.P. (1997), New method for retrospective detection of exposure to organophosphorus anticholinesterases. Application to alleged sarin victims of Japanese terrorists. *Toxicol. Appl. Pharmacol.* **146**, 156-161.

Raveh, L., Grunwald, J., Marcus, D., Papier, Y., Cohen., E., Ashani, Y., (1993) Human butyrylcholinesterase as a general prophylactic antidote for nerve agent toxicity, *Biochem. Pharmacol.* **45** (12), 2465-2474

Raveh, L., Grauer, E., Grunwald, J., Cohen, E., and Ashani, Y, (1997) The stoichiometry of protection against soman and VX toxicity in monkeys pretreated with human butyrylcholinesterase. *Toxicol. Appl. Pharmacol.* **145**, 43-53.

Rosenberg, Y., Luo, C., Ashani, Y., Doctor, B.P., Fischer, R., Wolfe, G. and Saxena, A., (2002) Pharmacokinetics and immunological consequences of exposing macaques to purified homologous butyrylcholinesterase, *Life Sci.* **72,** 125-134

Spruit, W.E.T, Langenberg, J.P., Trap, H.C., Van der Wiel, H.J., Helmich, R.B., Van Helden, H.P.M., and Benschop, H.P. (2000) Intravenous and inhalation toxicokinetics of sarin stereoisomers in atropinized guinea pigs. *Toxicol. Appl. Pharmacol.* **169**, 249-254.

Spruit, H.E.T., Trap, H.C., Langenberg, J.P. and Benschop, H.P., (2001) Bioanalysis of the enantiomers of (±)-sarin using automated thermal cold trap injection combined with two-dimensional gas chromatography, *J. Anal. Toxicol.* **25**, 57-61.

Sweeney, R.E. and Maxwell, D.M.(1999) A theoretical model of the competition between hydrolase and carboxylesterase in protection against organophosphorus poisoning, *Math. BioSci.,***160** , 175-190.

Sweeney, R.E. and Maxwell, D.M.(2003) A theoretical expression for the protection associated withj stoichiometric and catalytic scavengers in a single compartment model of organophosphorus poisoning, *Math. BioSci.,* **181**, 133-143.

Trap, H.C. (2002), Unpublished results of TNO Prins Maurits Laboratory.

Van Helden, H.P.M., Langenberg, J.P., and Benschop, H.P. (2000) Low level exposure to GB vapor in air: diagnosis/dosimetry, lowest observable effect levels, performance-incapacitation, and possible delayed effects. Final Report for Grant Agreement DAMD17-97-1-7360.

Van Helden, H.P.M., Trap, H.C., Oostdijk, J.P., Kuijpers, W.C., Langenberg, J.P., and Benschop H.P., (2003) Long term, low-level exposure of guinea pigs and marmosets to sarin vapor in air: lowest observable effect level, *Toxicol. Appl. Pharmacol.* **189**, 170-179.

Van der Schans, M.J., Langenberg, J.P., and Benschop, H.P. (2000) Toxicokinetics of O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothioate [(±)-VX] in hairless guinea pigs and marmosets – Identification of metabolic pathways. Final Report for Cooperative Agreement DAMD17-97-2-7001.

Van der Schans, M.J., Lander, B.J., Van der Wiel H., Langenberg, J.P., and Benschop, H.P., (2003) Toxicokinetics of the nerve agent (±)-VX in anesthetized and atropinized hairless guinea pigs and marmosets after intravenous and percutaneous administration, *Toxicol. Appl. Pharmacol.* **191**, 48-62.

Wolfe, A.D., Rush, R.S., Doctor, B.P., Koplovitz, I., and Jones, D. (1987) Acetylcholinesterase prophylaxis against organophosphate toxicity. *Fundam. Appl. Toxicol.* **9**, 266-270.

Wolfe, A.D., Blick, D.W., Murphy, M.R., Miller, S.A., Gentry, M.K., Hartgraves, S.L., and Doctor, B.P. (1992) Use of cholinesterase as pretreatment drugs for the protection of rhesus monkeys against soman toxicity. *Toxicol. Appl. Pharmacol.* **117**, 189-193.

IX APPENDIX 1 EVALUATION OF THE RATE CONSTANTS FOR INHIBITION OF HuBuChE BY TWO STEREOISOMERS OF AN ORGANOPHOSPHATE SHOWING A SMALL DIFFERENCE IN ANTICHOLINESTERASE ACTIVITY, FROM REACTION WITH ITS RACEMIC MIXTURE

Two parallel reactions proceed between enzyme (E) and the two stereoisomers of the organophosphate (I_+ and I_-)

$E + I_+ \rightarrow E\,I_+$
$E + I_- \rightarrow E\,I_-$

It is derived for these reactions that
$-d[E]/dt = k_1 [E] [I_+] + k_2 [E] [I_-]$ (1)
$-d[I_+]/dt = k_1 [E] [I_+]$ (2)
$-d[I_-]/dt = k_2 [E] [I_-]$ (3)

If parameter $\theta = \int_o^t [E]\,dt$ or $d\theta = [E]\,dt$ (4)

equations (1), (2) and (3) can be written as

$-d[E] = \{k_1 [I_+] + k_2 [I_-]\}\,d\theta$ (5)
$-d[I_+] = k_1 [I_+]\,d\theta$ (6)
$-d[I_-] = k_2 [I_-]\,d\theta$ (7)

After integration of equations (6) and (7) it is found that

$[I_+] = [I_{+,o}] \exp(-k_1 \theta)$ (8)
$[I_-] = [I_{-,o}] \exp(-k_2 \theta)$ (9)

where $[I_{+,o}]$ and $[I_{-,o}]$ are the initial concentrations of I_+ and I_-, respectively.

Substitution of equations (8) and (9) into equation (5) and integration of this equation leads to

$[E] = [E_o] + \frac{1}{2} [I_o] \{\exp(-k_1 \theta) + \exp(-k_2 \theta) - 2\}$ (10)

where $[E_o]$ and $[I_o]$ are the initial concentration of enzyme and total organophosphate, whereas $\frac{1}{2} [I_o] = [I_{+,o}] = [I_{-,o}]$.

The rate constants k_1 and k_2 were calculated from values of residual enzyme activities determined at various times of inhibition, as follows.

The relationship of residual enzyme concentration and time of inhibition is described as a fourth or fifth order polynomial calculated from the data determined for residual enzyme concentration, derived from the residual enzyme activities, at various times of inhibition:

$[E] = a + b\,t + c\,t^2 + d\,t^3 + e\,t^4 (+f\,t^5)$ (11)

Next, values of θ are calculated at the various times of inhibition at which residual enzyme activity had been determined, according to

$$\theta = \int_0^t [E]\,dt = \int_0^t (a + b\,t + c\,t^2 + d\,t^3 + e\,t^4)\,dt = a\,t + 1/2\,b\,t^2 + 1/3\,c\,t^3 + 1/4\,d\,t^4 + 1/5\,e\,t^5 \quad (12)$$

in case of a fourth order polynomial.

Finally, equation (10) is fitted to the set of data for [E] and θ obtained in this manner, from which values for k_1 and k_2 are obtained.

X APPENDIX 2 CALCULATION OF RELEVANT TOXIC LEVEL

In a manner similar to that applied earlier with regard to the toxicokinetics of soman, it can be derived up to which timepoint the concentration of nerve agent is toxicologically relevant. The reasoning is based on the assumption that subsequent to total inhibition of AChE at a critical site such as the diaphragm, 5-7% AChE activity should be reactivated by an oxime (or be released after protection by carbamate) without being reinhibited by residual nerve agent, in order to ensure survival. The concentration of AChE in the diaphragm of guinea pigs is estimated to be 2.6 nM. Therefore it is assumed that the reactivated fraction corresponds with 150 pM AChE. Based on the measured bi-molecular reaction constant between AChE from guinea pigs and VX (3e8 M^{-1} min^{-1}) it can be derived that this reactivated fraction of AChE can be re-inhibited by 175 pM VX (40 pg/mL) with a half life of ca 15 min. An order of magnitude lower concentration can cause only insignificant re-inhibition. In a more generalized way, it may be reasoned than an AUC of 0.040 * 15 = 0.6 ng.min.mL-1 in the last part of the toxicological curve is needed for toxicological relevance. In a similar way the AUC for the other nerve agents may be calculated.

Nerve agent	Averaged binding constant with AChE (M^{-1}.min^{-1})	Concentration AChE that is reactivated (pM)	Half life (min)	AUC (ng.min.mL^{-1})
(-)-VX	3e8	150	15.4	0.6
C(±)P(-)-soman	1e8	150	46	1.2
(-)-sarin	1.4e7	150	330	6.9

XI TECHNICAL OBJECTIVES/STATEMENT OF WORK

Toxicokinetic studies

1. Determination of the time course of HuBuChE activity in blood of guinea pigs after i.m. administration of the enzyme. A dose of HuBuChE will be used which results in a total amount of the enzyme in blood that is 0.7 times the dose corresponding with 2 LD50 (i.v.) of C(±)P(±)-soman, just prior to nerve agent administration.

2. Determination of the time courses of C(±)P(±)-soman stereoisomers and of HuBuChE activity in blood of guinea pigs after pretreatment with HuBuChE (i.m.; dose as for item 1) and intoxication with an i.v. bolus dose corresponding with 2 LD50 of the agent.

3. Determination of the time courses of C(±)P(±)-soman stereoisomers and of HuBuChE activity in blood of guinea pigs after pretreatment with HuBuChE (i.m.; dose as for item 1) and intoxication with a second i.v. bolus dose corresponding with 2 LD50 of the agent given 90 min after the first dose.

4. Determination of the time courses of (±)-sarin stereoisomers and of HuBuChE activity in blood of guinea pigs after pretreatment with HuBuChE (i.m.; dose as for item 1) and intoxication with an i.v. bolus dose corresponding with 2 LD50 of the agent.

5. Determination of the time courses of (±)-VX stereoisomers and of HuBuChE activity in blood of hairless guinea pigs after pretreatment with HuBuChE (i.m.; dose as for item 1) and intoxication with an i.v. bolus dose corresponding with 2 LD50 of the agent.

6. Determination of the time courses of C(±)P(±)-soman stereoisomers and of HuBuChE activity in blood of guinea pigs pretreated with HuBuChE (i.m.) and intoxicated by nose-only exposure during 2 min to a dose corresponding to 2 LCt50 of the agent. A dose of HuBuChE will be used which results in a total amount of the enzyme in blood that is ca. 0.25 times the dose corresponding with 2 LCt50 of C(±)P(±)-soman in guinea pigs, just prior to nerve agent exposure.

7. Determination of the time courses of C(±)P(±)-soman stereoisomers and of HuBuChE activity in blood of guinea pigs pretreated with HuBuChE (i.m., dose as for item 6) and intoxicated by nose-only exposure during 300 min to a dose corresponding to 2 LCt50 of the agent.

8. Determination of the time courses of (±)-sarin stereoisomers and of HuBuChE activity in blood of guinea pigs pretreated with HuBuChE (i.m., dose as for item 6) and intoxicated by nose-only exposure during 2 min to a dose corresponding to 2 LCt50 of the agent.

9. Determination of the time courses of (±)-VX stereoisomers and of HuBuChE activity in blood of hairless guinea pigs pretreated with HuBuChE (i.m., dose as for item 1) and intoxicated with a percutaneous dose corresponding with 2 LD50 of the agent.

10. Determination of the time course of HuBuChE activity in blood of marmosets after i.m. administration of the enzyme. A dose of HuBuChE will be used which results in a total amount of the enzyme in blood that is *ca.* 0.5 times the dose corresponding with 2 LCt50 of C(±)P(±)-soman in marmosets, just prior to nerve agent exposure.

11. Construction of a nose-only exposure chamber for marmosets.

12. Determination of the time courses of C(±)P(±)-soman stereoisomers and of HuBuChE activity in blood of marmosets pretreated with HuBuChE (i.m., dose as for item 10) and intoxicated by a 2-min nose-only exposure to an (estimated) dose corresponding with 2 LCt50 of the agent.

13. Determination of the time courses of C(±)P(±)-soman stereoisomers in blood of marmosets intoxicated by a 2-min nose-only exposure to an (estimated) dose corresponding with 0.8 LCt50 of the agent.

14. Isolation of C(+)P(+)- and C(-)P(+)-soman by treatment of C(+)P(±)- and C(-)P(±)-soman, respectively, with α-chymotrypsin and isolation of C(+)P(-)- and C(-)P(-)-soman by treatment of C(+)P(±)- and C(-)P(±)-soman, respectively, with rabbit serum.

15. *In vitro* determination of the rate constants for inhibition of HuBuChE by the single stereoisomers of C(±)P(±)-soman at pH 7.5 and 38 °C.

16. *In vitro* determination of the rate constants for inhibition of HuBuChE by the stereoisomers of (±)-sarin at pH 7.5 and 38 °C from kinetic analysis of inhibition experiments performed with the racemic mixture.

17. *In vitro* determination of the rate constants for inhibition of HuBuChE by the stereoisomers of (±)-VX at pH 7.5 and 38 °C from kinetic analysis of inhibition experiments performed with the racemic mixture.

18. Determination of the HuBuChE activities in blood and in homogenates of lung, liver, kidney and brain from guinea pigs pretreated with HuBuChE (i.m., dose as for item 1). Blood and tissues will be removed at the point of time after enzyme administration at which the nerve agent is administered in the toxicokinetic experiments (items 2-5).

19. Determination of the HuBuChE activities, total concentrations of bound C(±)P(±)-^{14}C-soman and concentrations of C(±)P(±)-^{14}C-soman bound to HuBuChE in blood and in homogenates of lung, liver, kidney and brain from guinea pigs pretreated with HuBuChE (i.m., dose as for item 1) and intoxicated with a dose corresponding with 2 LD50 (i.v.) of C(±)P(±)-^{14}C-soman. Blood and tissues will be removed 1 min after C(±)P(±)-^{14}C-soman administration.

20. Determination of the HuBuChE activities, total concentrations of bound C(±)P(±)-^{14}C-soman and concentrations of C(±)P(±)-^{14}C-soman bound to HuBuChE in blood and in homogenates of lung, liver, kidney and brain from guinea pigs pretreated with HuBuChE (i.m., dose as for item 1) and intoxicated with a dose corresponding with 2 LD50 (i.v.) of C(±)P(±)-^{14}C-soman. Blood and tissues will be removed 90 min after C(±)P(±)-^{14}C-soman administration.

21. Development of a physiologically based model for the i.v. toxicokinetics of C(±)P(±)-soman in guinea pigs pretreated with HuBuChE (i.m.) by introduction into the model of data obtained in the present studies on the distribution of HuBuChE over various tissues (items 18-20) and on the rate constants for inhibition of the stereoisomers of C(±)P(±)-soman (item 15). Data obtained from toxicokinetic studies (item 2) will be used for validation of the model.

IX LIST OF PERSONNEL RECEIVING PAY UNDER THIS COOPERATIVE AGREEMENT

Dr. Marcel J. van der Schans
Dr. Jan P. Langenberg
Dr. Carolien M. Boone
Dr. Henk P. Benschop
Dr. Daan Noort
Mr. Henk C. Trap
Mr. Kees Pleijsier
Mr. Alex Fidder
Mr. Ton L. van der Laaken
Mrs. Herma J. van der Wiel
Mrs. Brenda.J. Lander
Mrs. Helma E.T. Spruit
Mrs. Marjan J. Jongsma
Mrs. Corry van Dijk